The effect of modulating membrane lipid composition on the thermal sensitivity of tumour cells in culture

Kingston, Catharine Alison

The Effect of Modulating Membrane Lipid Composition on the Thermal Sensitivity of Tumour Cells in Culture

by

Catharine Alison Kingston
B.Sc. (Dunelm)

Being a thesis submitted for the degree of
Doctor of Philosophy
of
The University of Durham
October 1989

Dedicated to the memory of my mother
Wendy Kingston

Abstract

The plasma membrane, which separates intracellular contents from extracellular milieu, consists of a lipid bilayer comprising mainly phospholipids and cholesterol together with various functional proteins, which control the interaction of the cell with its environment. Cells are killed at elevated temperatures and previous work suggests that the plasma membrane may be a primary target in this hyperthermic cell death. The present study set out to test this hypothesis using a rat liver tumour cell line (Hepatoma Tissue Culture cells). Several different experimental approaches were adopted.

Supplementation of these cells with linoleic acid (18 : 2) for a 36 hour period increased the thermal sensitivity of cells at $43^{\circ}C$, though increased sensitivity was not evident at other times. Plasma membrane-enriched fractions were obtained from control cells and from cells supplemented with linoleic acid for a 36 hour period, then lipids were extracted and characterised. Whilst there was little difference in the cholesterol:phospholipid ratio, the phospholipid fatty acid composition of membranes from supplemented cells showed elevated levels of 18 : 2 and decreased levels of oleic acid (18 : 1) relative to control cell membranes. DPH fluorescence polarisation studies indicated that plasma membranes from supplemented cells were less 'ordered' than control membranes. Alkaline phosphodiesterase I, a plasma membrane-bound enzyme, appeared to be more thermolabile in supplemented cells suggesting that plasma membrane 'fluidity' may be an important factor in determining the thermal sensitivity of this membrane-bound enzyme.

Hyperthermic cell death was potentiated by the presence of local anaesthetics, two of which, dibucaine and tetracaine, also produced less 'ordered' membranes. Morphological studies conducted on cells in the presence and absence of

local anaesthetic at elevated temperatures indicated changes in cellular surface morphology on heating which were accelerated in the presence of the anaesthetic.

The intermediate filament network of these cells did not appear to be a primary target of hyperthermic treatment.

These studies suggest that the lipid composition and physical state of the plasma membrane are critical features involved in the expression of cell death, possibly through a modulation of membrane protein thermal sensitivity.

Acknowledgements

I would like to express my thanks to the North of England Cancer Research Campaign for providing financial support for the work undertaken in this thesis and to Professor K. Bowler for providing research facilities in the Department of Zoology.

I am very grateful to Professor K. Bowler and Dr. R. Manning for their help and advice during the course of the study and for their critical reading of the manuscript.

I am indebted to the following people for their technical support: Miss J. Chambers for her expert and invaluable assistance particularly in cell culture work; Mr T. Gibbons for general technical assistance; Mrs C. Richardson for Electron Microscopy work and Mr D. Hutchinson for photography.

I would also like to thank Dr. A. R. Cossins of Liverpool University for the use of Fluorescence Polarisation equipment and Dr. A. H. Seheult of Durham University for guidance in the statistical treatment of some of the data presented in this thesis.

Special thanks go to Shab Ladha and Kay Fogg for their moral support and friendship during our time together in the 'huts'.

I would like to thank my family and K.T. for their love and encouragement. Finally my love and greatest thanks go to Simon for the countless hours he has spent in the preparation of this thesis.

Contents

Declaration

I declare that this thesis is original. No part of it has been submitted previously for a degree at any other university. All work shown is my own except for the fluorescence polarisation measurements which were made by Professor K. Bowler at Liverpool University. The plasma membrane study was carried out jointly with Dr. R. Manning and S. Ladha.

Glossary

BHT	Butylated hydroxytoluene
BSA	Bovine serum albumin
Ca^{2+}-, Mg^{2+}-free PBS	Calcium and magnesium free phosphate buffered saline
CHO	Chinese hamster ovary
DPH	1,6-Diphenyl-1,3,5-hexatriene
EDTA	Ethylenediamine tetra-acetic acid
FFA	Free fatty acid
Hepes	N-2-Hydroxyethylpiperazine-N'-2-ethanesulphonic acid
HTC	Hepatoma tissue culture
Mg^{2+}ATPase	Magnesium-dependent, adenosine 5′-triphosphate
MTT	3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazoliumbromide
Na^{+}/K^{+}ATPase	Sodium and potassium stimulated magnesium-dependent adenosine 5′-triphosphate —— - ase
NBS	Newborn bovine serum
r_{av}	Average radius of rotation

Other abbreviations used are in accordance with the Biochemical Journal (Policy of the Journal and Instructions to Authors: (1989) *Biochem. J.* 257 1–21).

The notation used in the thesis to describe individual fatty acids refers to the carbon chain length and the number of unsaturated bonds. e.g. 18 : 2 refers to a fatty acid with a carbon chain length of 18 having two unsaturated bonds.

Materials

All reagents were of analytical grade unless stated otherwise.

Agar Aids, Stanstead, Essex.

Araldite

Dibutylphthalate

Dodecenylsuccinic anhydride (DDSA)

2,4,6-Tri(dimethylaminomethyl)phenol 30 (DMP 30)

Glutaraldehyde

Sodium cacodylate

Aldrich Chemical Co. Ltd., Gillingham, Dorset.

Isopropyl ether

Alltech/Applied Science, Carnforth, Lancashire.

10% Alltech CS-5, on a chromasorb WAW support (100-120 mesh)

GLC fatty acid methyl ester standards ($12:0 - 24:0$)

Amersham International plc, Amersham, Buckinghamshire.

[8-^3H] Adenosine 3′5′-cyclic phosphate ammonium salt

BDH Ltd., Poole, Dorset.

Acetic acid (glacial)

Ammonium molybdate

Butan-1-ol

Chloroform

Choloxidate No. 1 kit

Cupric sulphate

Deoxyribonuclease I from bovine pancreas (3.1.4.5)

Diethyl ether

Heptane

Hydrochloric acid

Light petroleum (b.p. 40-60°C)

Orthophosphoric acid

Paraformaldehyde

Phenylmethylsulphonylfluoride

PIPES

Potassium chloride

Propylene oxide

Ribonuclease I from bovine pancreas (3.1.4.22)

Sulphuric acid

Triton X-100

Zinc dibenzyldithiocarbamate (laboratory reagent)

B.O.C. Ltd., Vigo Lane, Birtley, Co. Durham.

Nitrogen

Air

Hydrogen

Coulter Electronic Ltd., Luton, Bedfordshire.

Isoton II

Fisons Scientific Apparatus, Loughborough, Leicestershire.

Imidazole

Methanol

Flow Laboratories Ltd., Rickmansworth, Hertfordshire.

Ca^{2+}-, Mg^{2+}-free PBS

Eagles Minimum Essential Medium

Fungizone (amphotericin B solution)

L-Glutamine

NBS

Non-essential amino acids

Penicillin

Sodium bicarbonate (7.5% solution)

Streptomycin

Tylosin

Trypsin 2.5% (w/v)

James Burrough (FAD) Ltd., Witham, Essex.

Absolute alcohol

JJ (Chromatography) Ltd., King's Lynn, Norfolk.

Glass g.l.c. columns, $2m$ long, $2mm$ internal diameter, $6mm$ external diameter

Kemox (UK) Ltd., Holton, Oxfordshire.

Osmium tetroxide

Pharmacia (GB) Ltd., Milton Keynes, Buckinghamshire.

Cytodex 2

Cytodex 3

Percoll

All other reagents were obtained from *Sigma Chemical Co., Poole, Dorset.*

Chapter I

General Introduction

Temperature has a profound influence on living organisms and their metabolic processes (Schmidt Nielson, 1982). Whilst it is a general and universal feature that animals are able to make adaptive responses to changes in environmental temperature (Cossins and Bowler, 1987), it is also true that damage and cell death occur, especially in mammalian cells, following exposure of cells to temperatures only a few degrees above the normal range (Burger and Fuhrman, 1964). For humans this normal range varies from 35.1° to 37.7°C, whilst the normal range for all other mammals lies between 34°C and 40°C.

Cancer cells are also sensitive to elevated temperatures (Levine and Robins, 1970; Giovanella et al., 1976). Cancer is a term that describes a situation in which one cell, any cell, acquires the properties of unrestrained proliferation and invasiveness and is able to bequeath these properties to its descendants through countless generations (Cameron and Pauling, 1979). Clinical hyperthermia, as a treatment for cancer relates to the artificial elevation of the temperature of the tissue above normal body temperature. For therapeutic purposes, treatment levels are generally in the range 42°C-45°C corresponding to elevations ranging between 5°C and 8°C. The objective of this temperature rise is the treatment of tumours either directly, by inducing irreversible biological damage by exploiting any differential thermal sensitivity of tumour cells that may exist, or indirectly by potentiating the effect of other well established treatment regimes such as surgery, radiotherapy or chemotherapy.

Using elevated temperatures to treat cancer is by no means a modern concept. A 'fire drill' used to burn away tumours in the breast is mentioned in the Egyptian papyrus discovered by Edwin Smith thought to date from 3000 BC (Breasted, 1930). Over the last 100 years or so apparently spontaneous remissions of cancer were noted following prolonged intense fevers due to a variety of

infections. For example, Busch (1866) noted that a facial sarcoma showed complete regression after two attacks of erysipelas associated with high fever. This led Coley (1893) to devise a hyperthermic treatment by deliberate production of fever by administering a preparation containing bacterial pyrogenic toxins. The toxin injection produced a pyrexia of $40°C$ for 4-6 hours and resulted in a number of dramatic responses in patients with inoperable and advanced malignant disease.

The use of heat as a treatment for cancer raises the question: are tumour cells more thermally resistant than normal cells? In several studies (Westermark, 1927; Jares and Warren, 1939; Allison and Paton, 1965; Auersperg, 1966; Levine and Robins, 1970; Muckle and Dickson, 1971; Giovanella et al., 1976) some of which were performed more than 50 years ago, it has been suggested that neoplastic cells are *in vitro* as well as *in vivo* more easily damaged by elevated temperatures than are normal cells. However, many studies of the *in vitro* heat sensitivity of tumour cells (Jares and Warren, 1959; Muckle and Dickson, 1971) have not correlated their observations on tumour cells with similar effects on normal cells, being more interested in the description of the phenomena or in the mechanism underlying them (Allison and Paton, 1965). In those studies in which a comparison between normal and tumour cells has been performed, the choice of material selected for the purpose is sometimes open to criticism. Bhuyan (1979) has argued that the value of studies conducted by Auersperg (1966) and Levine and Robins (1970) was limited since Auersperg (1966) compared non-growing fibroblasts with dividing epithelial carcinoma cells whilst Levine and Robins (1970) compared spleen cells with Ehrlich ascites cells. Similarly, studies by Harisiadas et al. (1975) compared survival of 'normal' liver cells with those obtained from a closely associated hepatoma and reported that hepatoma cells were slightly more resistant to heat than normal cells. Likewise, solid tumour physiology is now thought to be a major factor resulting in the apparent heat sensitivity of some tumour cells or subpopulations within a tumour. For example, most normal tissues when faced with a heat challenge react by increasing blood flow which serves to dissipate the heat. However, these normal physiological responses are often different or totally lacking in the neovasculature of tumours which permits the differential heating of normal tissues and tumours growing within normal tissue (Stewart, 1988). In addition, the extracellular milieu in

parts of most solid tumours has been shown to be characterised by nutrient depletion (Thomlinson and Gray, 1955), low *pH* (Wike-Hooley et al., 1984) and chronic hypoxia (Vaupel, 1979) largely as a result of an inadequate vascular supply (Otte, 1988). These conditions have been shown to markedly influence the thermosensitivity of mammalian cells in culture. Cell culture techniques are frequently used in biological studies since the physiological and physiochemical conditions can be regulated very easily. The use of cell culture techniques in the study of tumour cells is widespread since work suggests that tumour cells die in a similar manner both *in vivo* and *in vitro* with the major differences following hyperthermic treatment relating to events occurring after the cessation of the hyperthermic treatment such as *in vivo* immune responses and regional blood flow responses (Hahn, 1982).

The effect of *pH* on cellular thermal sensitivity has now been studied in at least ten different cell lines in culture (see Gerweck, 1988) and with the exception of an SDB rat mammary carcinoma cell line (Dickson and Oswald, 1976) thermal sensitivity has been found to be increased when the *pH* of the culture medium was reduced. The fact that *pH* sensitivity *in vitro* is manifest over a *pH* range which is observed in tumour tissue, i.e. *pH* 6.6 to *pH* 7.0 (Gerweck, 1977), attests to its likely significance *in vivo*. Similarly, several studies have compared the response of cultured cells to hyperthermia under normoxic and hypoxic conditions (Kim et al., 1975; Bass et al., 1978; Gerweck, 1979). Although varying results have been obtained, in general it appears that cells are equally or more sensitive to hyperthermia under hypoxic compared to normoxic conditions. Likewise *in vitro* studies by a number of workers (Kim et al., 1980; Gerweck et al., 1984) have shown that when cultured cells are exposed to reduced levels of both glucose and oxygen, thermal sensitivity is markedly increased. Taken together such results strongly suggest that variations in normal and tumour tissue cellular microenvironments could well play an important role in the differential heat sensitivity of many solid tumours that has been observed.

On the other hand, a number of rigorous and quantitative studies have suggested that some tumour cells are indeed more thermosensitive than their normal cell counterparts. For example, studies by Chen and Heidelberger (1969)

in which they compared mouse prostate cells 'transformed' *in vitro* by a carcinogenic hydrocarbon with normal prostate cells, and by Giovanella et al. (1976) in which they compared human colon carcinoma cells with interstitial epithelial cells and melanoma cells with melanocytes have suggested that tumour cells are more thermosensitive than normal cells based on their decreased cloning efficiency in culture and their decreased ability to produce tumours upon inoculation into an appropriate recipient.

Thus it would seem that although some tumour cells are more thermally resistant than normal cells this sensitivity is not a general characteristic of all malignant cells. It seems likely that all cells die by a similar mechanism but that some tumour cells may possess certain characteristics or exist in a particular microenvironment which renders them more sensitive to heat than their normal counterparts.

This leads on to the question of how and why do cells die when exposed to elevated temperatures? Exposure of mammalian cells to hyperthermic temperatures in the range 43-46°C has been found to cause a wide range of effects. For example, hyperthermia inhibits protein synthesis (Henle and Leeper, 1979), induces heat shock proteins (Killey and Schlesinger, 1978), alters membrane fluidity (Li and Hahn, 1980) and produces chromosomal aberrations (Dewey et al., 1971) which suggests that heat induced injury and death is probably the end result of interference with a variety of different intracellular sites and functions.

The vast majority of studies of *in vitro* responses to hyperthermia use the ability of cells to form colonies as the assay for injury since this provides an unambiguous definition of survival. Analysis of survival curves, produced by plotting the logarithm of the surviving fraction against time of heat treatment, following exposure of cells to elevated temperatures for various lengths of time has revealed that survival curves can be grouped into three classes according to the shape of the survival pattern. One class is exemplified by curves generated from HeLa cells heated at temperatures between 41°C and 45°C for periods of up to 5 hours (Gerner et al., 1975) where survival is exponential over the entire time range suggesting a direct correlation between cell death and time at hyperthermic temperature. The second and most usual type of survival curves are typified by

Chinese hamster ovary (CHO) cells heated at temperatures ranging from 43.5°C to 46.5°C (Westra and Dewey, 1971). These curves are characterised by an initial shoulder with the shape becoming log-linear after longer exposure times. Several workers have suggested that the shoulder on heat survival curves implies an ability of the cells to sustain sublethal damage (Hahn, 1982) though it has not, as yet, been possible to show experimentally whether or not recovery from sublethal damage or repair of sublethal lesions does occur in heated cells. A third type of survival curve is seen in Chinese hamster cells heated at temperatures below 43°C (Sapareto et al., 1978). Whilst the initial portion of the survival curve is characterised by a shoulder region followed by a log-linear region, at longer exposures (typically 3-4 hours) the curve flattens out ending in a 'resistant' tail. Several workers have shown that this 'resistant' tail or plateau indicates the development of thermal tolerance which occurs in some, but not all, cell lines (Henle and Roti Roti, 1988). Two types of thermotolerance have been described. The first, as suggested above, is induced by prolonged heating at temperatures below 43°C where the cells become more resistant to heat after 3 to 4 hours of hyperthermia whilst the second develops as a consequence of a short exposure to hyperthermic temperatures (41°C to 46°C) during subsequent recovery of the cells at physiological temperatures (37°C). Thermotolerance is expressed as an increase in cellular resistance to further heat treatment (Nielsen and Overgaard, 1979). This thermotolerance effect is short lasting and is lost after a few hours at 37°C. Whilst the molecular basis for this thermotolerance is not understood there are numerous reports that hyperthermia results in the synthesis of a specific set of proteins (heat stress or heat shock proteins) and there is some evidence that thermotolerance is related to such protein synthesis (e.g. Burdon, 1985).

Other *in vitro* studies of hyperthermia have revealed that cellular heat sensitivity can vary enormously between specific mammalian cell lines even though cells are grown and heated under the same culture conditions. Raaphorst et al. (1979) have shown that cell survival of mouse LP59 cells after 5 hours at 42.5°C was 10^4 times lower than the survival of pig kidney CCL33 cells. These results suggest that some cells must possess certain characteristics which make them more vulnerable to the effect of heat than other cells. Also, the fact that cell lines derived from the same tissue, e.g. human melanoma xenografts in different individuals, can span a range of heat sensitivities (Rofstad and Brustad, 1984)

indicates that it is not possible to predict the heat sensitivity of specific cell lines based on their tissue of origin. Another interesting finding is that the differential heat sensitivity of two cell lines at one specific temperature may not be applicable for predicting their relative heat sensitivity to other temperatures. Raaphorst et al. (1979) showed that whilst Muntjac (Indian deer) CCL157 cells and pig kidney CCL33 cells showed similar heat sensitivities at $42.5°C$, after 80 minutes at $45.5°C$ cell survival of the Muntjac cells was more than 10^3 times lower than the survival of the pig kidney cells suggesting the complex nature of the response of cells to hyperthermia.

Because of the complex nature of the hyperthermic response of cells, quantitation of 'thermal dose' in a biologically meaningful way so that hyperthermal treatments can be compared is extremely difficult. Whilst radiation workers use the energy deposited to describe the dose of radiation since this relates directly to the resulting effect, the situation with hyperthermia is rather different since the biological response is primarily dependent on the time at an elevated temperature and not on the deposition of energy (Hahn, 1982). If the temperature was fixed and constant, time at the raised temperature would be a perfectly reasonable method of defining a thermal dose. However, the temperature is not fixed and in clinical practice is certainly far from constant. Variations in temperature and time, as already mentioned, can have a marked effect in determining the overall biological response leading to phenomena such as thermotolerance. Consequently, there is as yet no fully satisfactory method of defining thermal dose. This problem has been examined recently by Field (1987 a,b).

Jung (1986) has proposed an interesting mathematical model for cell killing based on the analysis of many survival curves obtained after hyperthermic treatments of Chinese hamster ovary (CHO) cells at various temperatures or after consecutive exposure to two different temperatures. He has suggested that cellular inactivation by heat is a two step process in which heat serves to cause nonlethal lesions which are then converted into lethal lesions upon further heating. It is assumed that both the production and conversion of nonlethal lesions occur at random and it is suggested that cell death results from the conversion of one of the nonlethal lesions.

Bowler (1987) has proposed an interesting descriptive model for cell killing in which he suggests that heating perturbates function at a primary site(s) leading to an impairment of function at that site which in turn affects the functioning at secondary sites causing their malfunction. This 'knock-on' effect of damage at one site causing malfunction at other sites is envisaged to lead to a cascade and accumulation of damage which at some point causes an irreversible loss in function at some site(s) resulting in cell death. The major differences between the model of Jung (1986) and that of Bowler (1987) is that whilst Jung (1986) believes that heat produces nonlethal lesions which are converted into lethal lesions, with time, to bring about cell death, Bowler (1987) believes heat produces primary lesions which may or may not be, or become, irreversible that cause a wide range of secondary knock-on effects which become irreversible with time leading to cell death.

To examine the feasibility of such models for cell killing has required the development of techniques such as cinematography, dye exclusion and monolayer detachment for use in post heating studies. Whilst clonogenic assays of the type developed by Puck and Marcus (1956) indicate that hyperthermia causes a dose dependent fraction of cells to be killed, such assays give no information regarding the events which accompany cell death and lysis. Zielke-Temme and Hopwood (1982) and Coss and Dewey (1988) have used time lapse cinematography to observe the time course of cell death in CHO cells heated in the G_1 phase of the cell cycle. These studies have led these workers to the conclusion that heat can kill by more than one mechanism, since cells either died prior to division or after having divided irregularly. Zielke-Temme and Hopwood (1982) suggested that less severe heat induced a mitotic-linked death whilst more severe heat induced an interphase death. Vidair and Dewey (1988) who followed CHO cells heated in plateau phase for up to 6 days post heating have suggested that heat-sterilised cells die by one of two modes of death which are distinguished by both the timing and type of the expressed damage. A rapid mode of death occurs during the first few days post heating and is characterised by cell detachment and inhibited rates of protein, RNA and DNA synthesis, whilst a slow mode of death becomes evident after cell detachment has ceased and after the cells have fully recovered from the heat induced inhibition of macromolecular synthesis. Cells exhibiting the slow mode of death were found to have reduced plating efficiencies relative

to non-heated populations and contained a large fraction of cells with multiple nuclei (Vidair and Dewey, 1988).

However, despite a wealth of observations and data on the effects of hyperthermia and models such as those of Jung (1986) and Bowler (1987) that have been proposed to account for cellular heat injury and death, the mechanism of cell death at elevated temperatures is still unknown. For example, the studies of Vidair and Dewey (1988) have certainly suggested that the slow mode of death is unlikely to be due to an irreversible inhibition of protein, RNA or DNA synthesis since all these processes recover in cells. However, the results are unable to establish what the primary site of damage is likely to be or whether the two modes of death result from similar damage differing only in severity or rather from different initial types of damage. The major problem is that temperature has an all pervasive influence on cellular structures so that heat damage is likely to occur at numerous sites, which makes it difficult to determine what factors might be of primary importance in the hyperthermic killing process. Most of the major cellular structures have been implicated as having a significant role in heat injury.

One of the earliest 'targets' implicated in hyperthermic damage were lysosomes. Because lysosomes are thought to be involved in cell lysis it was suggested that hyperthermia killed cells by causing lysosome rupture which resulted in the cell being digested by lysosomal enzymes. This idea was supported by the observation that proteolytic activity increased 1.5-fold following exposure of PNJ tumour cells to $42.5°C$ for 1 hour (Overgaard and Poulsen, 1977). Additional support came from histochemical studies of mouse spleen which showed increased acid phosphatase activity following hyperthermia (Hume and Field, 1977). However, the fact that agents which are known to enhance the susceptibility of lysosomal membranes to damage such as trypan blue, retinol and hydrocortisone did not affect cell killing by heat (Hofer et al., 1979) argues against the lysosome hypothesis. In addition, since lysosomes are involved in the destruction of dead cells many of the above considerations could well reflect secondary rather than primary effects of heat damage.

Heat induced damage to mitochondrial membranes has been suggested as

another primary target of hyperthermia. For example, Wheatly et al. (1989) reported marked changes in the ultrastructure of HeLa S_3 cell mitochondria which appeared early on during hyperthermic treatment and correlated well with the loss of viability and metabolic functioning found after treatment. This led these workers to suggest that mitochondrial effects are intimately and possibly primarily involved in heat induced damage. Similar findings have been reported by a number of other workers. For example, Welch and Suhan (1985) observed more swollen mitochondria and greater intacristal spaces in rat fibroblast cells after heat treatment whilst Borrelli et al. (1986) reported dense mitochondrial granules in synchronous G_1 CHO cells which were more prominent after heating. However, Heine et al. (1971) working with HeLa S_3 cells found no significant changes in the mitochondria of cells heated for several hours at $45°C$.

Similarly, whilst heat induced damage to mitochondrial membranes should result in measurable changes in cellular respiration rates, data in the literature concerning the rates of both respiration and glycolysis are quite contradictory. For example, Strom et al. (1977) working with a number of rat hepatomas and human melanomas and osteosarcomas reported that glycolysis was unaffected by exposure of cells from these tumours to temperatures up to $44°C$. Dickson and Suzanger (1976) however found that temperatures as low as $42.5°C$ served to inhibit glycolysis in human tumours. Results obtained from respiration studies are also controversial. Whilst Strom et al. (1977) working with Novikoff hepatoma cells, which were exposed to a temperature of $43°C$ for up to 4 hours reported only a slight decline in oxygen rates measured at $38°C$ for up to 6 hours following heat exposure, Durand (1978) working with V-79 cells recorded very different results. Whilst cells incubated at temperatures above $42°C$ showed an initial increase in oxygen uptake this was short lived and was followed by a sharp decline in respiration. The duration of the increase and the subsequent rate of decline were found to be temperature dependent. In addition, the sharp decline in respiration was found to precede the initiation of cellular inactivation by a few minutes suggesting that cells became extremely heat sensitive once their metabolic rate and hence presumably their rate of ATP production was reduced. Work by Laval and Michel (1982) has suggested that ATP levels may well play a key role in thermal sensitisation since cells which were treated with inhibitors of ATP synthesis showed increased thermal sensitivity and decreased levels of ATP.

However, more recent work by Calderwood (1987) suggests that a general role for energy in cellular responses to heat is unlikely. He found that heat induced lesions in energy production were not reflected by changes in parameters of energy status in most of the cells studied. For example, he reported that over 99% of HA-1 fibroblasts were killed by $45°C$ heat before a decrease was observed in any parameter of energy status.

Another mechanism that has been put forward to explain hyperthermic cell death is inactivation of cellular proteins. There is certainly some evidence to suggest that one or more cellular proteins may be critical elements at elevated temperatures. For example, cells incubated in the presence of sulfhydryl-rich compounds become very heat sensitive (Kapp and Hahn, 1979). Likewise, the fact that the activation enthalpy for cell killing above $45°C$ is similar to that observed for protein denaturation (Johnson et al., 1974) suggests that protein denaturation could be involved in cell killing. In addition, the finding that cells may be protected against heat damage by deuterium oxide (D_2O) and glycerol (Fisher et al., 1982) has been thought to be indicative of protein involvement in cell killing. It has been suggested that the deuterium is substituted for hydrogen and this stabilises the protein against heat, or that a solvent effect occurs that increases the strength of the hydrophobic interactions. However, whilst glycerol and dimethylsulphoxide also form hydrogen bonds, only glycerol protects against heat damage. In addition, although several studies have shown that protein synthesis is almost completely inhibited by the exposure of cells to $43°C$ or higher (Henle and Leeper, 1979), these studies have also shown that some time after the return of cells to $37°C$ protein synthesis resumes, which suggests that inhibition of protein synthesis is unlikely to be the cause of cell killing.

Another target of heat induced damage that has been suggested is the nucleus which contains the major portion of the cells genetic information encoded in DNA. It is not thought that the DNA molecule itself is the direct target for heat inactivations since the melting temperature of DNA *in vitro* is around $87°C$. In addition, there is little evidence implicating DNA synthesis since Henle and Leeper (1979) have shown that although DNA synthesis is greatly reduced after exposure of cells to elevated temperatures this inhibition is reversible when cells are returned to normal temperatures. However, there is some evidence for a role

of chromosomes in heat death since Dewey et al. (1971) reported that S phase cells heated to $45°C$ developed chromosomal aberrations and that the number of these aberrations correlated with cell death. Similarly, Tomasovi et al. (1978) have shown that DNA isolated from heated cells is rich in nonhistone proteins and that the amount of bound protein is a function of thermal dose. However, this observation raises the question is this protein-DNA binding a direct effect of hyperthermia? If heat is acting specifically within the nucleus to modify the rate of protein binding to DNA one might expect the excess protein to be located within the nucleus. However, since Roti Roti and Winward (1980) found no increase in the protein content when isolated nuclei were heated at $45°C$ for 30 minutes it seems unlikely that protein-DNA binding has a causal relationship to cell inactivation. The primary effect of heat in this instance would not appear to be the binding itself but rather modification of the nuclear envelope or some other extranuclear event. Similarly, the fact that a number of workers (e.g. Wong and Dewey, 1982) have described heat induced strand breaks in DNA which only occur during the post heating incubation period suggests that such changes could well be a result of cell necrosis rather than a direct effect of hyperthermic treatment.

By far the greatest number of studies attempting to elucidate the effects of heat on mammalian cells have shown some modification of membrane structure and/or function (Hahn, 1982). Since the plasma membrane is important in regulating ion transport, cell recognition, receptor mediated processes, energy transduction and also acts as a barrier to diffusion (Lee and Chapman, 1987) it is perhaps to be expected that it will be affected by hyperthermia in a variety of ways.

There is indeed considerable evidence to support the idea that the plasma membrane is involved in cell killing. Agents that are known to perturb the plasma membrane, including local anaesthetics and aliphatic alcohols, all act synergistically with heat (Yatvin, 1977; Li and Hahn, 1978). In addition, the effect that these agents produce by themselves on cells is very similar to the action of heat alone (Li et al., 1980). Some morphological evidence exists suggesting that membrane structure is disrupted by exposure to elevated temperatures. For example, a discontinuous plasma membrane has been observed by Schrek et al.

(1980) in heated lymphocytes, whilst the loss of microvilli (Mulcahy et al., 1981) is a common observation in heat damaged cells. Many workers (e.g. Bass et al., 1982; Borrelli et al., 1986) have also reported extensive blebbing in heat treated cells with the degree of membrane blebbing showing a direct correlation with the degree of cell death.

Further evidence for an important role of the plasma membrane in the hyperthermic death of cells comes from membrane permeability studies. Hyperthermia has been found to alter the membrane permeability to several compounds including adriamycin (Hahn and Strande, 1976), polyamines (Gerner et al., 1980) and certain ions (Strom et al., 1977). Under physiological conditions animal cells contain a high intracellular concentration of potassium and low intracellular concentrations of calcium and sodium as compared with extracellular fluids. Any impairment of the control of permeability at hyperthermic temperatures will allow the leak of K^+ from and Ca^{2+} and Na^+ into cells. Both Yi et al. (1983) and Ruifrock et al. (1985) have found that heating lowers intracellular $[K^+]$. Vidair and Dewey (1986) on the other hand reported no change in intracellular levels of Na^+, K^+ or Mg^{2+} using a heat dose that caused a 98% reproductive death of CHO cells. Vidair and Dewey (1986) have suggested that these different results may reflect the metabolic state of the cells with uncontrolled ionic exchanges only occurring in metabolically 'dead' cells. A number of reports have suggested that cells accumulate Ca^{2+} during hyperthermia (Anghileri et al., 1985; Wiegant et al., 1985; Stevenson et al., 1986). Since intracellular calcium is known to play a key role in the control of many aspects of cellular activity, e.g. cell proliferation (Whitfield et al., 1976) and enzymatic phosphorylation and dephosphorylation (Westwood et al., 1985), a change in calcium levels could lead to a loss of cellular homeostasis and eventually to cell death. However, as yet there is little agreement as to whether the rise in calcium that is observed is actually involved in the cell killing process due to a number of discrepancies that exist between studies (see Bowler, 1987).

Hyperthermia at $43.5°C$ and $45°C$ has been found to inhibit the uptake of thymidine in CHO cells grown in suspension and as monolayers (Slusser et al., 1982) whilst exposure of rat and human thymocytes to temperatures ranging from $39°C$ to $43°C$ resulted in striking inhibition of Na^+ dependent amino acid

transport (Kwock et al., 1978; Lin et al., 1978). This entry mechanism is believed to be a membrane protein mediated process which suggests that hyperthermia could result in a rearrangement of membrane protein conformation.

Understanding why the plasma membrane might be sensitive to heat requires an understanding of membrane structure. The widely accepted model of biological membranes today is a modification of the fluid-mosaic model proposed by Singer and Nicholson (1972) with lipid molecules forming an asymmetric bilayer which also contains integral and peripheral proteins and cholesterol. The hydrophilic polar head groups of the phospholipids that comprise membranes are oriented towards the outer surfaces of the bilayer, whilst the hydrocarbon tails are found in the interior. The molecular motion of phospholipids containing saturated fatty acyl chains has been found to be restricted when measured by techniques such as electron spin resonance or fluorescence polarisation (Alberts et al., 1983), whilst the molecular motion of phospholipids containing unsaturated fatty acyl chains has been found to be less restricted.

Phospholipids in artificial bilayers undergo phase changes often near $20°C$. Below this so called 'phase transition temperature' the lipids are in a solid 'gel phase' whilst above this temperature lipid bilayers enter a more fluid, 'liquid crystalline' phase. In mammalian cell membranes phase changes are masked by the presence of cholesterol and proteins. Cholesterol acts as a buffer of fluidity, stiffening the membrane at temperatures above the phase transition and fluidising it below that temperature, whilst the presence of proteins in the plasma membranes leads to non-uniformity in the viscosity of lipids.

A possible link between growth temperature and the stability of cellular membranes was first suggested by Heilbrunn (1924) who proposed that resistance to heat was related to the melting temperature of the lipids. With an understanding of membrane structure, subsequent *in vitro* studies have shown that microorganisms (Sinensky, 1974) and ectothermal animals (Cossins and Sinensky, 1984; Hazel, 1984) respond to environmental temperatures by altering the degree of saturation of the fatty acyl chains of their cellular membrane phospholipids. Decreasing the growth temperature results in an increase in unsaturation, whilst an increase in growth temperature results in a more saturated membrane lipid frac-

tion. This phenomenon, termed 'homeoviscous adaptation' by Sinensky (1974), in which cells regulate membrane 'fluidity' (order) in a compensatory fashion, clearly highlights the functional importance of lipid fluidity to cells. Implicit in this strategy is that there is an optimal range of membrane fluidity for normal cell function (Bowler, 1987; Yatvin et al., 1987). The demonstration of homeoviscous adaptation suggests that one of the key factors determining a cell's response to hyperthermia could well be the physical state of its plasma membrane.

Lee and Chapman (1987) have reported that a change in temperature has two effects on membrane lipids. Firstly, an increase in temperature will result in kinetic energy causing an increase in the molecular motion of the membrane lipids. Secondly, an increase in temperature may well cause a change in the phase of the lipids from a more ordered gel phase to a less ordered liquid crystalline phase. The overall effect will be a progressive increase in fluidity, i.e. a decrease in order of the lipid molecules which could have important consequences for membrane protein-lipid interactions. Whilst membrane proteins affect the viscosity of the lipid bilayer, they themselves are likely to be influenced by the dynamic state of the membrane lipids by their packing and movements which will depend amongst other things on their thermal disorder. Cossins et al. (1981) have suggested that the anisotropic hydrophobic core of the membrane forms a relatively hindered, viscous environment for enzyme functioning and that as a consequence enzymes may well possess relatively loose tertiary structures to enable molecular flexibility that is vital for catalysis.

An increase in temperature that leads to an increase in fluidity would therefore provide a less hindered environment for the membrane proteins and could result in the proteins adopting configurations that are inactivating. Evidence in support of this idea comes from the studies of Cossins et al. (1981) who demonstrated that the Na^+/K^+ATPase from warm acclimated goldfish synaptic membranes were more thermally stable than the same enzyme from more fluid cold acclimated membranes (Bowler, 1987). Barker (1985) showed that Mg^{2+}ATPase in two transplantable rat tumours (MC7 and D23) was far more thermolabile than the corresponding enzyme from rat liver and that this heat sensitivity corresponded with lipid fluidity with the tumour membrane lipids showing a higher degree of disorder. Similarly, Cheng et al. (1987) have shown

that the thermostability of Ca^{2+}ATPase in reconstituted lipid vesicles can be correlated with the level of cholesterol, a component known to modulate the fluidity of the plasma membrane, again suggesting the importance of lipid 'fluidity' in determining membrane protein activity.

Since the 1960's when it was first realised that fairly extensive membrane lipid modifications could be produced both *in vivo* and *in vitro* by varying the composition of the bathing fluid surrounding cells (Geyer, 1962), many studies have adopted this approach to investigate the role of plasma membrane composition on heat induced cell killing. Many of these studies have provided support for the hypothesis that a cell's response to hyperthermia is related to its membrane fluidity at the time of treatment. For example, the fatty acid auxotroph *Escherichia coli* K1060 requires unsaturated fatty acids for growth and by varying the unsaturated fatty acids in the growth medium membrane composition can be markedly altered. On exposure to mild hyperthermia the sensitivity of *E.Coli* K1060 was found to increase in proportion with increased unsaturation index of the cellular lipids (Yatvin, 1977). Similarly, growing L1210 leukaemia cells in media supplemented with highly polyunsaturated fatty acids led to increased thermosensitivity, while decreased thermosensitivity was observed when cells were supplemented with more saturated fatty acids (Guffy et al., 1982). Lepock et al., (1981), on the other hand, concluded that there was a lack of correlation between hyperthermic cell killing and membrane fluidity based on their experiments with the anti-oxidant compound butylated hydroxytoluene in a V79 Chinese hamster cell line. They proposed that a correlation exists between membrane protein denaturation and hyperthermic killing (Massicotte-Nolan et al., 1981; Lepock et al., 1983) rather than membrane fluidity and hyperthermic killing.

Other workers have considered the importance of cholesterol in the hyperthermic response. The fact that cholesterol is an important lipid molecule of the plasma membrane of eukaryotic cells and is known to modulate the physical properties of membranes (Oldfield and Chapman, 1972), acting as a membrane stabiliser (Sabine, 1983) suggests that cells with a higher cholesterol content will be more heat resistant because the higher the level of cholesterol that is present in membranes, the less will be the increase in membrane fluidity. This hypothesis

was first tested by Cress and Gerner (1980) who measured the cholesterol content of five mammalian cell lines and reported a positive correlation between heat sensitivity at $43°C$ and cholesterol levels when expressed on the basis of cellular protein. However, comparable investigations that have since been performed by other groups have failed to confirm Cress and Gerner's results. Anderson et al. (1985) and Konings and Ruifrok (1985) examined the cholesterol/protein ratios of cells in a number of mammalian cell lines and found little differences in this ratio but marked differences in heat sensitivity. In addition, Konings and Ruifrok (1985) also showed that when the three cell lines used were adapted to a different nutrient medium, less cholesterol was present in the cells but the heat sensitivity did not alter. Anderson et al. (1984) found that in heat resistant variants of B16 melanoma cells the cholesterol/phospholipid ratio and cholesterol/protein ratio decreased rather than increased with increasing heat resistance. Similarly, Raaphorst et al. (1985) failed to show a consistent relationship between cholesterol content and thermal sensitivity of normal and X-ray transformed $C3H\ 10T\frac{1}{2}$ mouse embryo cells. However, the significance of many of these cholesterol studies is questionable since the purity of the cell fractions used for cholesterol assays is not always reported.

The work produced over the past few years has contributed greatly to our picture of the changes occurring in the heated cell and would certainly seem to imply a major role for the plasma membrane in the hyperthermic killing process. The main aim of the work undertaken in this thesis therefore was to clarify further the significance of the plasma membrane, and in particular the lipid composition of the plasma membrane, in the hyperthermic death of cells and to investigate the possibility that a cell's response to hyperthermia is related to its membrane fluidity at the time of treatment.

In order to do this, the membrane lipid composition of rat liver tumour cells maintained in culture was modified by dietary supplementation of specific lipids in the culture medium and the thermal sensitivity of control and supplemented cells was investigated over a range of hyperthermic temperatures ($42.5°C$ to $45°C$). Changes in lipid composition, particularly with respect to the fatty acid composition of phospholipids, and the level of cholesterol were monitored after the isolation of plasma membranes from control and supplemented cells. Physical

measurements, using steady state fluorescence polarisation were employed to establish whether any change occurred in the fluidity of plasma membranes derived from supplemented cells. In addition, surface morphological studies were carried out in an attempt to correlate changes in membrane structure with hyperthermia. Furthermore, the molecular basis of hyperthermia was investigated by assessing the effect of any changes in membrane lipid composition and fluidity on the thermal sensitivity of plasma membrane-bound enzymes (e.g. Na^+/K^+ATPase, alkaline phosphodiesterase I).

To investigate further the importance of membrane 'fluidity' (order) in hyperthermic cell death, the role of local anaesthetics, known to fluidise membranes, was also examined by surface morphological studies and steady state fluorescence polarisation techniques. In addition, the effect of heat on intermediate filaments (IF), one of the three fibrous systems of the cytoskeleton, was examined. The IF system is thought to serve as a mechanical integrator of cellular space (Lazarides, 1980). Since intermediate filaments have been found in close association with a number of subcellular components including the plasma membrane, it was of interest to see how they were affected by the presence of heat in the presence or absence of anaesthetic.

It is hoped that such studies will contribute to our knowledge of the cause of thermal sensitivity and hence offer possible methods for its potentiation, thus making hyperthermia a more useful clinical tool as a treatment for cancer.

Chapter II

Characterisation of the Culture System for HTC Cells

2.1 Introduction

Studies of tumour cells have often involved the growth of solid tumours in continuous passage in laboratory animals (Upreti et al., 1983; Boddie et al., 1985). However, such systems have a number of drawbacks. Solid tumours invariably contain a heterogeneous population of cells. Also the properties of passaged tumours often show considerable change with time (Steel, 1977). When hyperthermic studies are performed with animals bearing tumours it is difficult to control experimental conditions precisely. For example, low pH, nutrient depletion and chronic hypoxia characterise the extracellular milieu in parts of most solid tumours (Samulski et al., 1984).

Cell culture techniques have successfully overcome many of the problems associated with the growth of solid tumours. The practice of culturing cells in isolation away from the controlling and modifying influences of other cells within an organism dates back to Baker (1933), who successfully cultivated fowl monocytes in glass flasks. Initiation of a cell culture involves mechanical or enzymatic dispersion of a tissue into a cell suspension. These cells are then cultured either as an adherent monolayer, on a solid substrate, or as a suspension in an appropriate culture medium. Cell proliferation within such cultures means that they, in turn, can be dispersed by enzymatic treatment or simple dilution and reseeded into fresh vessels producing characteristic cell lines.

A major advantage of cell culture is that it permits considerable control of the physicochemical environment in terms of pH, temperature, osmotic pressure, and oxygen and carbon dioxide tensions. In addition, the physiological conditions can also be kept relatively constant, though not necessarily defined, since most media still require the presence of serum which is highly variable in its composition (Honn et al., 1975). This means that cells *in vitro* can be manipulated in many

ways that cannot be achieved with cells *in vivo*. Cultured cells also exhibit a very high degree of homogeneity which means that replicate samples are virtually identical. In addition, the use of cultured cells in experiments is more economical than rearing and performing experiments with intact animals.

The choice of cell to be used in any study will be influenced by a number of factors including the nature of the proposed work, the type of culture required and the quantity of cells. Plasma membrane modifications and hyperthermic studies have been performed on a wide variety of cell lines including L1210 murine leukaemia cells (Symonds et al., 1981; Guffy et al., 1982; Burns et al., 1986) which grow in suspension and have a doubling time of approximately 12 hours; HeLa cells (Burdon et al., 1982; Cress et al., 1982; Kampinga et al., 1988; Wheatley et al., 1989) some of which grow in suspension (e.g. HeLa S_3 cells) and some of which grow as monolayers (e.g. HeLa human cervical carcinoma cells) with doubling times in the range 20 to 26 hours; and Chinese Hamster Ovary (CHO) cells (Henle and Leeper, 1976; Borrelli et al., 1986; Bates and Mackillop, 1987; Chu and Dewey, 1987) which can be grown in suspension or as monolayers and have a doubling time of 13 to 15 hours.

The use of the Hepatoma Tissue Culture (HTC) cell line in the present study partly reflects the earlier interest shown in this laboratory in rat liver tumours (Barker, 1985) and also reflects the use of the cell line by other workers. Clearly in a study such as this, in which the effects of modulating membrane lipid composition on the thermal sensitivity of tumour cells in culture are to be examined, some background information is desirable. Schamhart et al. (1984) have investigated cell survival and cell morphology of HTC cells following exposure to temperatures between $37°C$ and $44°C$ and have shown a remarkable difference in thermal sensitivity between this cell line and the H35 hepatoma cell line originating from the same tissue. A number of other workers have already looked at various aspects of lipid metabolism in HTC cells. For example Wood and Falch (1973) performed a quantitative determination of phospholipid class compositions and fatty acid compositions of individual phosphoglycerides derived from HTC cells grown on media containing varying levels of serum and lipids. Alaniz et al. (1975, 1976, 1982, 1984), Wiegand and Wood (1974), Gaspar et al. (1975, 1977) and Marra et al. (1984) have looked at the incorporation, biosynthesis and

metabolism of a wide range of fatty acids in HTC cells. Whilst Lopez-Saura et al. (1978) and Sauvage et al. (1981) have also characterised and purified the plasma membrane of HTC cells by cell fractionation techniques. Such studies therefore form a useful starting point for the current investigations.

One of the biggest problems facing cell culture is contamination. The media for the cultivation of cells is highly nutritious not only for animal cells, but also for bacteria, fungi and mycoplasma. These generally have a much faster growth rate than the animal cells and can produce toxins that are lethal to them. Since decontamination, especially with mycoplasmas, is difficult to achieve and can produce hardier, antibiotic-resistant strains of the contaminant, the general rule is that contaminated cultures are discarded. As a result, one of the most important aspects of cell culture is the avoidance of contamination by the growth of cells in aseptic conditions.

There are many potential sources from which contamination can arise including the atmosphere in which culture procedures are performed, the apparatus, the culture medium and the operator. Consequently all apparatus and liquids that come into contact with cultures are routinely sterilised either by autoclaving or by filtration. The adoption of good aseptic technique within a culture unit is a major way of preventing contamination. Correct aseptic technique provides a barrier between the micro-organisms in the environment outside the culture and the pure uncontaminated culture within the culture vessel.

Culture medium is often supplemented with antibotics as an extra safeguard against contamination. A wide range of antibiotics have been developed over the years. Streptomycin and penicillin are commonly used to give protection against gram-positive and gram-negative bacteria (Nielsen and Overgaard, 1979; Von Hoff et al., 1986). Other antibiotics used against bacteria include gentamycin (Schamhart et al., 1984; Guffy et al., 1982) and neomycin (Silvestrini et al., 1983). Amphotericin B deoxycholate has been employed against fungal and yeast contamination (Pearlman, 1979). A number of agents including tylosin (Friend et al., 1966) and more recently, ciprofloxacin (Schmitt et al., 1988) have been used against mycoplasma.

Although pathogen-free cell culture is difficult in the absence of such antibiotics, it is important to bear in mind that dependence on such agents may lead to microbial resistance, and does not necessarily prevent cryptic contamination. In addition some antibiotics can exert toxic effects at elevated temperatures (Hahn et al., 1977).

One of the major distinctions between cell culture systems is whether cells are grown attached to a substrate (monolayer culture) or in suspension (suspension culture). Suspension cultures derive from cells that can survive and proliferate without attachment. Monolayer culture however, implies that adherence is an integral part of survival and subsequent proliferation. In order to develop successful monolayer culture techniques it is important to understand the nature of the cell growth involved. Typically, the growth kinetics of cells in monolayer culture can be divided into three characteristic stages (McAteer and Douglas, 1979). Firstly, freshly seeded cells will experience a quiescent period (lag phase) during which there is no cell division. The cells then enter a log phase of growth in which there is an exponential increase in cell number. When the culture conditions can no longer support cell division the cells enter a stationary phase during which cell number remains constant.

There are many factors that will influence the length of these various stages such as the type of cell, the media composition (Richter et al., 1972; Porro et al., 1986), seeding density, pH, oxygen tension (Richter et al., 1972), surface area available for growth and the way in which cells are harvested. Puck et al. for example, in 1955, found that the then standard methods used for the trypsinisation and washing of cells (Dulbecco and Vogt, 1954; Youngner, 1954) subjected them to considerable trauma that greatly impaired their ability to initiate growth. Procedures adopted in cell culture studies aim to optimise growth by a careful consideration of such factors.

In monolayer culture, since the cell yield is proportional to the available surface area, the choice of culture vessel is generally determined by the number of cells required and the type of experiment. Very small numbers of cells can be grown in multiwell plates which offer small surface areas for growth and the possibility of multiple replicates. Larger numbers of cells are frequently grown in

petri dishes, or flasks, offering surface areas between $25cm^2$ and $175cm^2$. Flasks of various sizes have proved useful as the culture vessel in a wide range of experimental systems. Increasing the cell yield any further, which is necessary if, for example, biochemical analysis of cell plasma membranes is to be performed, either means increasing the size and number of conventional vessels or using suspensions of particulate microcarriers on which the cells can attach and multiply.

The idea of culturing anchorage-dependent cells on small spheres (microcarriers) kept in suspension by stirring, was first conceived by Van Wezel (1967). The great advantage of the microcarrier system is the very large surface area to volume ratio that it offers. Stirred microcarrier cultures can typically yield two to four times as many cells for a given volume of medium as compared to other monolayer culture techniques. The superior yields with microcarrier culture have been reported for a wide variety of systems including chicken fibroblasts (Mered et al., 1980). In addition the microcarrier system provides a much more homogeneous environment for the cell population due to the stirring which reduces the time-dependent changes in microenvironment that normally occur in traditional monolayer culture (Thilly and Levine, 1979).

In order for a microcarrier to be suitable for animal cell culture its surface properties must be such that cells can adhere with a degree of spreading which permits proliferation. Similarly, it should be non-toxic and non-rigid. The latter characteristic helps to reduce the possibility of damage to the microcarrier and cells on stirring. The density should be slightly greater than the surrounding medium to facilitate its separation from the medium. In addition, the size distribution of microcarriers should be narrow so that even suspensions can be produced and cell confluency reached at approximately the same time on each microcarrier.

A wide range of materials have been used as microcarriers including porous silica, polystyrene plastic, polyacrylamide and glass (Varani et al., 1986). One of the most versatile forms of microcarrier is based on cross-linked dextran which has been derivitised into three types of microcarriers, namely Cytodex 1, Cytodex 2, Cytodex 3 (Pharmacia Fine Chemicals, 1981).

Cytodex 1 and Cytodex 2 possess specific densities of small charged molecules to promote attachment of cells. In Cytodex 1 the positively charged groups are distributed throughout the microcarrier matrix, whilst in Cytodex 2 there is only a surface layer of positively charged groups, in an attempt to reduce the binding of medium components, metabolites and cell products to the inner microcarrier matrix. In Cytodex 3 attachment of cells is promoted by the connective tissue protein collagen, since this has also proved to be a valuable cell culture substrate (Elsdale and Bard, 1972). Each microcarrier consists of a surface of denatured collagen that is bound to the cross-linked dextran matrix. Denatured collagen is used to overcome the specificity some cells show for attachment to particular forms of native collagen.

Although cells can often be grown on more than one type of microcarrier, the choice of Cytodex carrier used often reflects the purpose of the culture. Cytodex 1 is particularly useful for general purpose microcarrier culture and has been employed for the successful cultivation of more than 60 different cell types including primary cells, diploid cell strains and established or transformed cell lines (Clark and Hirtenstein, 1980). Cytodex 2 has been found to be advantageous where the production of viruses or cell products is desired, whilst Cytodex 3 is commonly used for cells known to be difficult to grow in culture. In addition, because the denatured collagen layer is susceptible to digestion by a variety of proteases, including trypsin and collagenase, Cytodex 3 is often used when it is necessary to remove cells from the microcarrier with the maximum possible recovery, viability and preservation of membrane integrity. Sirica et al., (1979), for example, obtained 100% recovery of rat hepatocytes from a collagen surface within 10 minutes using a collagenase solution.

The successful growth of cells in microcarrier culture depends on many interrelating factors. The initial phase of microcarrier culture is usually the most crucial (Clark and Hirtenstein, 1980) and must be carefully controlled. Increasing cell density, reducing culture volume and reducing stirring speed during this initial period can greatly improve cell growth and yield. (Pharmacia Fine Chemicals, 1981).

Once a microcarrier culture has been initiated conditions must be optimised

to maintain proliferation of cells so that maximum cell yields can be achieved. The rate at which a culture is stirred after initiation can markedly influence the growth and final yield (Hirtenstein and Clark, 1980). Rapid cell division and high cell densities lead to depletion of the medium components, to a decrease in culture pH, and often to a build up of metabolites such as ammonia and specific inhibitors (Cooper et al., 1959). Thus, the frequency and extent of medium replenishment is important. The ideal replenishment scheme is one which results in the smallest fluctuation of nutrient concentrations and pH during the culture cycle. In a closed microcarrier culture system where vessels are sealed and the supply of gas is only renewed when the culture is opened for sampling or replenishment of the medium, the culture volume within the vessel can influence the maximum yield of cells obtained from the culture (Hirtenstein et al., 1981). A reduction in cell yield has frequently been found to occur in a closed vessel that is more than half full. It is thought that such a reduction probably reflects the decreased supply of oxygen and, where CO_2-bicarbonate buffering systems are involved, the reduced head space volume available for buffering purposes.

In culture, because cells are being continuously cultivated in an artificial environment, they may lose characteristics apparent *in vivo* or gain characteristics that were not apparent (Coriell, 1979). Such alterations may include chromosomal aberrations (Chu et al., 1958) and changes in membrane structure and permeability (McAteer and Douglas, 1979). The likelihood of these occurrences has been found to increase with the age of the culture. As a precaution against such changes, and as a safeguard against accidental loss of cells by contamination, cell preservation techniques have been developed (Scherer and Hoogasian, 1954; Swim et al., 1958). These techniques involved glycerol as a cryoprotectant with storage in dry ice at $-70°C$. However, although cell cultures could be recovered in a viable state for many months, there was a gradual loss of viability at $-70°C$. Today, cell preservation involving the storage of frozen cell cultures in liquid nitrogen ($-196°C$) has become a standard practice in the culture process.

The potentially lethal effects of the freezing process have been minimised in a number of ways, including the addition of substances (e.g. glycerol, dimethyl sulphoxide) that lower the freezing point, the adoption of slow cooling rates that permit water to move out of the cell before it freezes and the storage of frozen cells

below $-130°C$ to retard the growth of ice crystals. In addition, rapid thawing of frozen cells has meant that the cell culture passes quickly through the $-50°C$ to $0°C$ temperature zone, where most cell damage is thought to occur.

The choice of cryoprotectant used in the freezing process and its concentration in the freezing medium depends on the cell type in many cases. For example, epithelial cells of human skin have been found to survive best in 20-30% glycerol whilst fibroblasts from the same tissue survive best in 10% glycerol (Athreya et al., 1969). Ashwood-Smith (1985) has reported the successful storage of bacterial strains for up to 11 years at $-196°C$ in the presence of 10% dimethysulphoxide (DMSO). However, where cells that are to be frozen show the possibility for gene activation and differentiation, it is currently thought to be advisable (Ashwood-Smith, 1985) to avoid the use of DMSO as a number of workers (Rudland et al., 1982; Higgins et al., 1983) have demonstrated gene activation effects of DMSO in a variety of cellular systems.

The aim of the current chapter is to introduce the techniques that were required for the successful culture of the chosen cell line and to account for the methods used in the study.

2.2 Materials and Methods

2.2.1 Cell Type

A rat liver hepatoma cell line known as the Hepatoma Tissue Culture (HTC) cell line was employed in this study. This line was first established from primary cultures of male Buffalo rats containing two lines of hepatoma in the ascites form (Thompson et al., 1966). Histologically, Thompson found that these HTC cells had the characteristics of 'epitheloid' cells, showing irregular cytoplasmic projections when growing in contact with glass and isolated from other cells, but becoming more rounded as intercellular contact was established. They were shown to exhibit logarithmic growth with a doubling time of approximately 24 hours.

In the present study HTC cells of unknown passage number were purchased from Flow Laboratories. The cells were grown as monolayers on plastic and

occasionally glass under sterile conditions. The growth medium used was Eagles Minimum Essential Medium (EMEM), developed initially in 1959 (Eagle, 1959). This EMEM purchased with Earles salt and non-essential amino acids but without glutamine and sodium bicarbonate, was supplemented with Newborn Bovine Serum (10%, v/v), $2mM$ glutamine, penicillin ($200IU/ml$), streptomycin ($200\mu g/ml$), tylosin ($10\mu g/ml$) and fungizone ($2.5\mu g/ml$) and was buffered at pH 7.4 with sodium bicarbonate ($2.0\ g/l$). The cells were maintained in a humidified incubator at $37°C$ in air/CO_2 ($19:1$, v/v).

2.2.2 Culture Techniques

Stock supplies of HTC cells were grown in either $25cm^2$ or $175cm^2$ flasks in $10ml$ or $70ml$ respectively, of the above growth medium. The medium was changed on alternate days and cells were subcultured when subconfluent i.e., when a sheet of cells covered approximately 80% of the total flask growth area. Stock flasks were seeded so that they required subculturing once a week on average. Generally, only cells that had been subcultured fewer than ten times in the laboratory were used in experimental studies.

(a) Cell harvesting and subculture

The medium was discarded from the flasks. Cells in each $25cm^2$ flask were washed twice with $10ml$ calcium- and magnesium-free phosphate buffered saline (Ca^{2+}-, Mg^{2+}-free PBS) and harvested using either 0.25% (w/v) trypsin in 0.2% (w/v) ethylenediamine tetra-acetic acid (EDTA) in Ca^{2+}-, Mg^{2+}-free PBS (trypsin-EDTA solution) or $2mM$ EDTA in Ca^{2+}-, Mg^{2+}-free PBS (EDTA solution). When trypsin-EDTA solution was used it was poured onto the cells ($1ml/25cm^2$ flask), swirled around and poured off. However, when the EDTA solution was used it was poured onto the cells ($3ml/25cm^2$ flask) and the flasks were placed in the $37°C$ incubator for 3 to 5 minutes. As soon as the cells began to round up and detach from the flask bottom, growth medium ($10ml/25cm^2$ flask) was added to the flasks to resuspend the cells, and the suspension was transferred to a sterile plastic universal tube. The cells were then spun down at $320g$ ($r_{av} = 16cm$) in a Mistral 3000 centrifuge at $20°C$ for 6 minutes. The pellet was resuspended in a small known volume of growth medium (typically 1 to $3ml/25cm^2$ flask) by three aspirations with a syringe fitted with a number 25

gauge hypodermic needle. The cells were then counted either by haemocytometer or Coulter Counter, before being used in experiments or to set up further stock flasks. The medium was always changed the day after subculturing.

(b) Microcarrier culture

Cell production was increased by microcarrier cell culture technology using Techne MCS microcarrier stirrer systems. Each stirrer system consisted of up to four glass culture vessels (Techne flasks), each of 1 litre capacity, in which cells were grown on microcarrier beads, maintained in suspension by a magnetically driven, bulb-shaped stirring rod which provided a gentle and even circulation of the microcarriers in the medium. The Techne flasks were maintained at $37°C$ ($\pm 1°C$) by a water bath positioned over the stirrer platform.

Microcarrier cell culture methods were developed from those given in a handbook entitled "Microcarrier cell culture : principles and methods" (published by Pharmacia Fine Chemicals). In the final method adopted, $1.5g$ of dry Cytodex 2 microcarriers per Techne flask were swollen in $75ml$ of Ca^{2+}-, Mg^{2+}-free PBS for a minimum of three hours at room temperature. The supernatant was decanted and the microcarriers washed twice with $50ml$ of fresh Ca^{2+}-, Mg^{2+}-free PBS.

The microcarriers were then sterilised by autoclaving at $110°C$ for 20 minutes at $15 p.s.i.$ After settling, the supernatant was discarded and the beads rinsed once with $50ml$ of warm growth medium, then suspended in $150ml$ of growth medium at $37°C$ and transferred to a siliconised sterile Techne flask. The flask was gassed with air/CO_2 ($19 : 1$, v/v) at $37°C$ for 15 to 30 minutes. Meanwhile, cells from two $175cm^2$ subconfluent stock flasks were harvested using trypsin-EDTA solution and counted by Coulter Counter.

Cells (4×10^7) were inoculated into the Techne flask which was gassed for a further ten minutes, sealed and transferred to the $37°C$ bath of the microcarrier stirrer system. Continuous stirring was commenced at $20 r.p.m.$ for 3 hours. After this time a further $100ml$ of growth medium was added and the flask was gassed again for 10 to 15 minutes. Stirring was increased to $30 r.p.m.$ when the flask was returned to the stirrer system. The microcarrier culture volume was

made up to $500ml$ with growth medium the following day and the flask was gassed for 15 minutes.

The Techne flask was cultured for up to five further days from the day of initiation, depending on the speed of cell growth. Growth of cells was monitored visually on the microcarrier beads by withdrawing representative samples and examining the beads by light microscopy. Each day during the culture period flasks were gassed for 15-30min at $37°C$ and $100ml$ of the supernatant was replaced with fresh growth medium.

Cells were recovered from the beads by the harvesting procedure detailed in Chapter 4, Materials and Methods, section 4.2.1(b).

Electron Microscopy Studies

HTC cells were cultured on Cytodex 2 microcarrier beads in a Techne flask as described above for a 72 hour period. The techniques used for the fixation of the HTC cells on beads were based on the methods of Karnovsky (1965).

Karnovsky fixative reagents

Solution A:

$2g$ paraformaldehyde and $40ml$ of distilled water. The paraformaldehyde was warmed with the distilled water with shaking and the precipitate that formed was dissolved by slowly adding 2-6 drops of $1M\ NaOH$.

Solution B:

$10ml$ of 25% (w/v) glutaraldehyde and $50ml$ of $0.2M$ sodium cacodylate, mixed together.

Solutions A and B were kept separate at $4°C$ and mixed in equal proportions immediately before use.

Aliquots $(0.75ml)$ of bead suspension were removed from the Techne flask and placed in plastic microfuge tubes (volume $1.7ml$). An equal volume of Karnovsky fixative was then added to each microfuge tube. After mixing the contents of the microfuge tubes the beads were allowed to settle and the supernatant was

removed. A further $1ml$ of Karnovsky fixative was added to the microfuge tubes which were placed in a rotator set at $2r.p.m.$ at $4°C$ for 1 hour. The Karnovsky fixative was then replaced with $1ml$ osmium tetroxide (1% w/v) in sodium cacodylate ($0.1M$) buffer, pH 7.3, for 0.5-1 hour at $4°C$.

After post-fixation the bead material was dehydrated at room temperature through an alcohol series of 70% (v/v), 90% (v/v) and absolute ethanol with 3 changes at each stage and for a total of 15 minutes at each concentration.

For Scanning Electron Microscopy (SEM) studies the alcohol was then substituted by acetone, for critical point drying purposes. Critical point drying prevents surface damage that often occurs when tissue is allowed to dry in air or under vacuum. The bead material in the microfuge tubes was taken through an acetone series of 50% (v/v), 70% (v/v) and 100% acetone at room temperature with 3 changes at each stage and for a total of 15 minutes at each concentration. Once the cells on the beads had been dried through the CO_2 critical point in an E3100 Jumbo critical point drier, the bead material was then sputter coated with gold-palladium in an E5100 SEM Polaron Coating Unit for examination in a Stereoscan 800 scanning electron microscope.

For Transmission Electron Microscopy (TEM) studies, following dehydration, the bead material was embedded in araldite in the following way.

Araldite mixture:

> $10ml$ of araldite (CY212), $10ml$ dodecenylsuccinic anhydride (DDSA), $1ml$ dibutylphthalate and $0.5ml$ 2,4,6-Tri(dimethylaminomethyl)phenol 30 (DMP 30).

The absolute alcohol in the microfuge tubes was replaced with a mixture of $0.5ml$ absolute alcohol and $0.5ml$ of propylene oxide and left for a total of 30 minutes with 3 changes of the mixture during this time. This mixture was then replaced with $1ml$ of propylene oxide for 30 minutes which was again changed 3 times. Following exposure to propylene oxide, the bead material was infiltrated for 30 minutes at $45°C$ in a mixture consisting of $0.5ml$ propylene oxide and $0.5ml$ of araldite. This was replaced with $1ml$ of araldite mixture which was changed after a further 30 minutes at $45°C$. The bead material was then left for

12 hours in this araldite solution at $45°C$ before being transferred to $60°C$ for a further 2 days.

The embedded bead material was sectioned on a Reichart OMU3 ultramicrotome using glass knives. Thin sections were doubly stained with uranyl acetate (1%, w/v) in 70% ethanol followed by lead citrate, prepared as described below, to give contrast to the sections.

lead citrate solution:

> $1.76g$ Tris-sodium citrate and $1.33g$ lead citrate were placed in a $50ml$ flask and $30ml$ distilled water was added. The solution was shaken for one minute then intermittently for 20 minutes. After shaking, $8ml$ $NaOH$ ($1M$) was added and the flask was inverted. The solution was then made up to $50ml$ with distilled water.

Three drops of uranyl acetate were placed on 'parafilm' with the clean side up. The grid bearing the sample sections was placed copper side down on the surface of the drop and left for 10 minutes. The grid was then washed by dripping distilled water over it for about 40 seconds and then dried carefully. After drying the process was repeated with lead citrate in place of uranyl acetate. the grid was then washed thoroughly again for about 40 seconds with distilled water. The sections were then examined in a Phillips 400T transmission electron microscope.

(c) Cryopreservation

Subconfluent cells from $2 \times 175cm^2$ flasks were harvested and the cell pellet obtained was resuspended in $10ml$ freezing medium (Foetal Bovine Serum / Glycerol; 92 : 8, v/v). Aliquots ($1ml$) of cell suspension in plastic vials were wrapped in cotton wool and placed in a polystyrene box. After sealing the box was maintained at $-80°C$ for a minimum of four hours, then the vials were transferred to canes and stored in liquid nitrogen at $-196°C$ for up to 26 months.

(d) Thawing

Vials of cells were thawed quickly by semi-immersion in a beaker of water at $37°C$. Cells were then added to $9ml$ of pre-warmed, pre-gassed growth medium in a $25cm^2$ flask and returned to culture. The medium was changed the next day

and cells were not used in experiments until they had been subcultured at least once.

2.2.3 Determination of Cell Number and Cell Size

(a) Cell size studies

Exponentially growing HTC cells were harvested from three $25cm^2$ flasks using trypsin-EDTA solution and resuspended in $5ml$ of Isoton II, a filtered phosphate-buffered saline solution. Using a Watson-Barnet stage micrometer and a calibrated eye piece on a Zeiss microscope, a sample of 100 cells were then examined and their diameters recorded.

(b) Haemocytometer cell counts

Counts of both live and dead cells were made initially by means of a haemocytometer using standard procedures. Solutions of harvested cells were prepared in a Trypan blue solution (0.6% w/v Trypan Blue, 0.85% w/v sodium chloride) such that when the suspension was added to the counting chamber of the haemocytometer approximately 50 to 100 cells were observed over each of the 9 large squares of the counting chamber. A minimum of two counts were performed on each cell sample.

(c) Coulter Counter cell counts

Although the haemocytometer counts provided quantitative information on cell viability, they were extremely time consuming to perform and began to impose serious limitations on the number of experiments that could be run. For this reason a Model D (Industrial) Coulter Counter, which was capable of counting and sizing up to 5000 cells per second, was purchased half way through the study.

A Coulter Counter determines the number and size of particles suspended in an electrically conductive liquid by forcing the suspension to flow through a small aperture that has an immersed electrode on either side. As a particle passes through the aperture it changes the resistance between the electrodes, which in turn produces a voltage pulse that is proportional in its magnitude to the particle

size. The series of pulses that are produced are then electronically scaled and counted.

Accurate measurements were obtained with the Coulter Counter by following a few simple guidelines. Firstly Isoton II (a filter sterilised buffered saline solution) was chosen as the electrolyte, since it was compatible with the characteristics of the cells, allowing true cell size measurements to be recorded. Secondly it was important to select an orifice tube with the correct size of aperture. Ideally the expected maximum particle size should not exceed approximately 40% of the aperture diameter. The cell sizing study enabled this criterion to be met. Also, since the Coulter Counter will only give a size analysis of the particulate material presented to the aperture, it was important to disperse the cells very well prior to counting, which was achieved by aspirating cell suspensions 3 times using a syringe fitted with a hypodermic needle (gauge number 25).

Once the Coulter Counter had been calibrated using smooth particles of known densities, a size distribution of HTC cells was established in the following way. Three $25cm^2$ flasks of exponentially growing cells were harvested and resuspended in $9ml$ of growth medium. A small volume ($2ml$) of this resuspension was added to $250ml$ of Isoton II in a plastic beaker and placed, after stirring, under the orifice tube of the Coulter Counter.

Now

$$equivalent\ particle\ volume = t \times I \times A\ ,$$

where

$$t = threshold \qquad I = aperture\ current \qquad A = attenuation$$

and

$$particle\ diameter = k\sqrt[3]{V}\ ,$$

where

$$k = calibration\ constant \qquad V = volume\ .$$

Thus, the total number of cells present could be calculated by setting the threshold (t), the aperture current (I) and the attenuation (A) to their lowest values and taking a cell count.

To obtain an accurate value of cell number, it was important that this maximum count did not exceed 10 310 with the $140\mu m$ aperture used, so that it was possible to apply a correction factor to compensate for the phenomenon of 'coincidence'. Coincidence occurs at high particle concentrations when two or more particles pass through an aperture simultaneously.

In addition, for statistical accuracy, it was necessary to take six counts for numbers less than 100, four counts for numbers from 100 to 1000 and two counts for numbers over 1000.

By gradually increasing the attenuation, threshold and aperture current, a range of cell size counts was made. A background count was then obtained for the electrolyte at each size level used for the sample analysis. The maximum acceptable level for this background count under these conditions was 150 counts.

(d) Comparison of haemocytometer and Coulter Counter methods

If the two methods available for cell counting were to be used interchangeably in future studies they needed to produce comparable results. A clonogenic assay (Kingston et al., 1989), first developed by Puck (1955) was used to assess their comparability.

Exponentially growing HTC cells were harvested using trypsin-EDTA solution and cell counts performed by haemocytometer and by Coulter Counter at threshold, aperture and attenuation settings determined by the earlier size distribution studies. A number of flasks were each seeded with 200 cells in $10ml$ of growth medium for each method of counting. Media changes were performed the following day (day 1) and then on day 5 and day 8. On day 9, the culture medium was discarded and cells were fixed in $10ml$ of Ca^{2+}-, Mg^{2+}-free PBS/methanol ($1:3$, v/v) for 10 minutes. After discarding this fixative, cells were stained with $5ml$ of 0.6% (w/v) Trypan blue in Ca^{2+}-, Mg^{2+}-free PBS for 10-15 minutes. After this time the number of cell colonies containing more than 50 cells (Von Hoff et al., 1986) as determined by microscopic observation, were counted.

2.2.4 Experimental Systems

(a) Flasks

The clonogenic assay described above (section 2.3(d)) was used routinely with $25cm^2$ flasks to assess cell number whenever small numbers of cells (< 800 per flask) were involved. Where large numbers of cells were present in $25cm^2$ flasks cell number was determined by harvesting the cells, either with trypsin-EDTA or EDTA solution and counting the cells by haemocytometer or Coulter Counter.

(b) Plates

The current study employed 24 well plates with $2.01cm^2$ growth area per well and a $2ml$ capacity. Cells for plates were derived from stock flasks containing exponentially growing cells by the usual harvesting procedure using EDTA solution. Wells were seeded with up to 5000 cells in $1ml$ of growth medium. The medium was changed the day after seeding and every two days thereafter and cells were cultured in plates for a maximum of 6 days.

The determination of cell number in plates was carried out by the colorimetric method of Mosmann (1983) with a number of modifications (Kingston et al., 1989). This assay is based on the cleavage of the tetrazolium salt, MTT, (3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) into a blue coloured product (formazan), by the mitochondrial enzyme succinate dehydrogenase (Slater et al., 1963). This conversion only takes place in living cells and the amount of formazan produced is reported to be proportional to the number of cells present (Green et al., 1984; Denizot and Lang, 1986).

In the final method adopted, MTT was dissolved at $5mg/ml$ in $20mM$ Hepes buffer (pH 7.4). This solution was sterilised by passage through a $0.22\mu m$ filter, which removed small amounts of insoluble residue, then it was diluted with an equal volume of $2\times$ concentrated phenol red-free, serum-free, fungizone-free growth medium, to produce a final MTT concentration of $2.5mg/ml$ (MTT medium).

The 24-well plates to be assayed were inverted and blotted to remove the growth medium (Denizot and Lang, 1986). Then $200\mu l$ of MTT medium was

added to each well and the plates were incubated at $37°C$ in an atmosphere of air/CO_2 ($19:1$, v/v) for 4 hours. Dimethylsulphoxide ($1ml$) was added to each well and mixed thoroughly to dissolve the dark blue crystals. Two $100\mu l$ samples were transferred from each well to a 96 well plate using a multichannel pipette and the absorbance was read on a Titertek Multiskan MCC/340 plate reader using a test wavelength of $540nm$ and a reference wavelength of $690nm$ (Denizot and Lang, 1986).

2.3 Results

2.3.1 Determination of Cell Size

Figure 2.1 shows the results of the cell sizing studies performed with a calibrated micrometer. It can be seen that the majority of diameters recorded lie between $10\mu m$ and $28\mu m$. The mean cell diameter recorded was $19.37\mu m$. In a normal distribution the median value is the same as the mean value. Since the median value for cell diameter was $19.74\mu m$ it can be seen that the cell diameters recorded approximate to a normal distribution. Some variation in cell size would be expected throughout the cell cycle. A cell after division will often be only half the size of one about to divide. It seems likely that the larger cell diameters observed were of cells about to undergo cell division whilst the smaller diameters were of cells that had just been produced by mitosis. For a normal distribution 95% of the population lies within ($\pm 2S.D.$) of the mean, whilst 99% of the population lies within ($\pm 3S.D.$) of the mean. Analysis of the data reveals that 92% of the population lies within the range 9.2 to $29.6\mu m$ ($\pm 2S.D.$) whilst 100% of the population lies within the range 4.1 to $34.7\mu m$ ($\pm 3S.D.$). The results would therefore suggest a normal distribution.

Figure 2.2(a) shows the cell size distribution data resulting from the Coulter Counter studies. The nature of the curve reveals that there is a single peak in the cell diameters recorded and that it occurs around about the $20\mu m$ range (indicated by the steepness of the gradient in this area). It can be seen that approximately 90% of cell diameters lie between $7\mu m$ and $34\mu m$. Reducing the diameter measurement below $6.97\mu m$ would appear to have little effect on the cumulative weight % oversize figure, suggesting that there were very few cells

Figure 2.1

Cell size distribution of HTC cells

Figure 2.1 shows the cell diameter distribution for 100 HTC cells. Exponentially growing cells were harvested using trypsin-EDTA solution and resuspended in Isoton II. A sample of 100 cells was then examined and their diameters recorded using a calibrated Watson-Barnet stage micrometer.

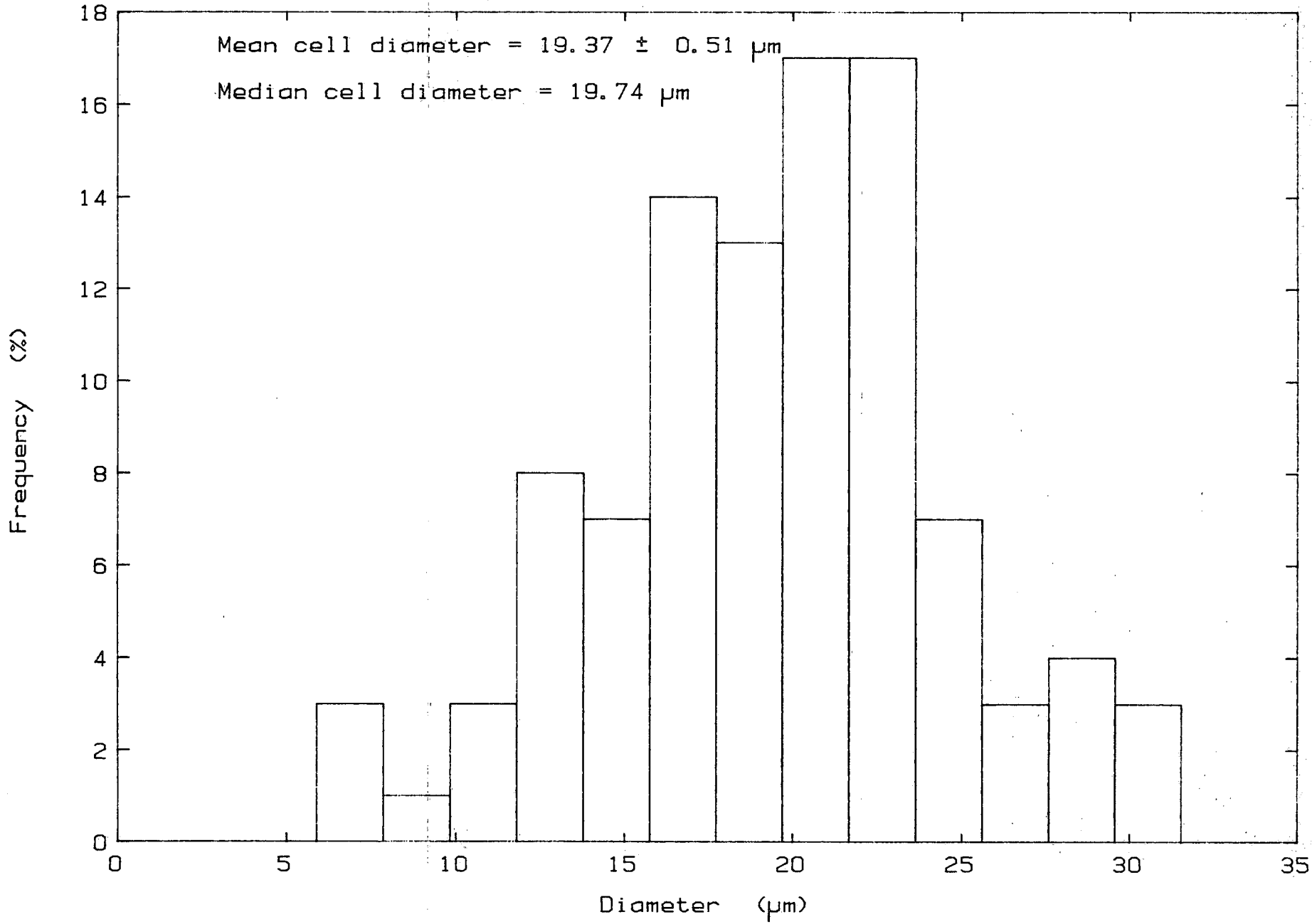

Mean cell diameter = 19.37 ± 0.51 µm
Median cell diameter = 19.74 µm
Frequency (%)
Diameter (µm)
0
2
4
6
8
10
12
14
16
18
0
5
10
15
20
25
30
35

Figure 2.2

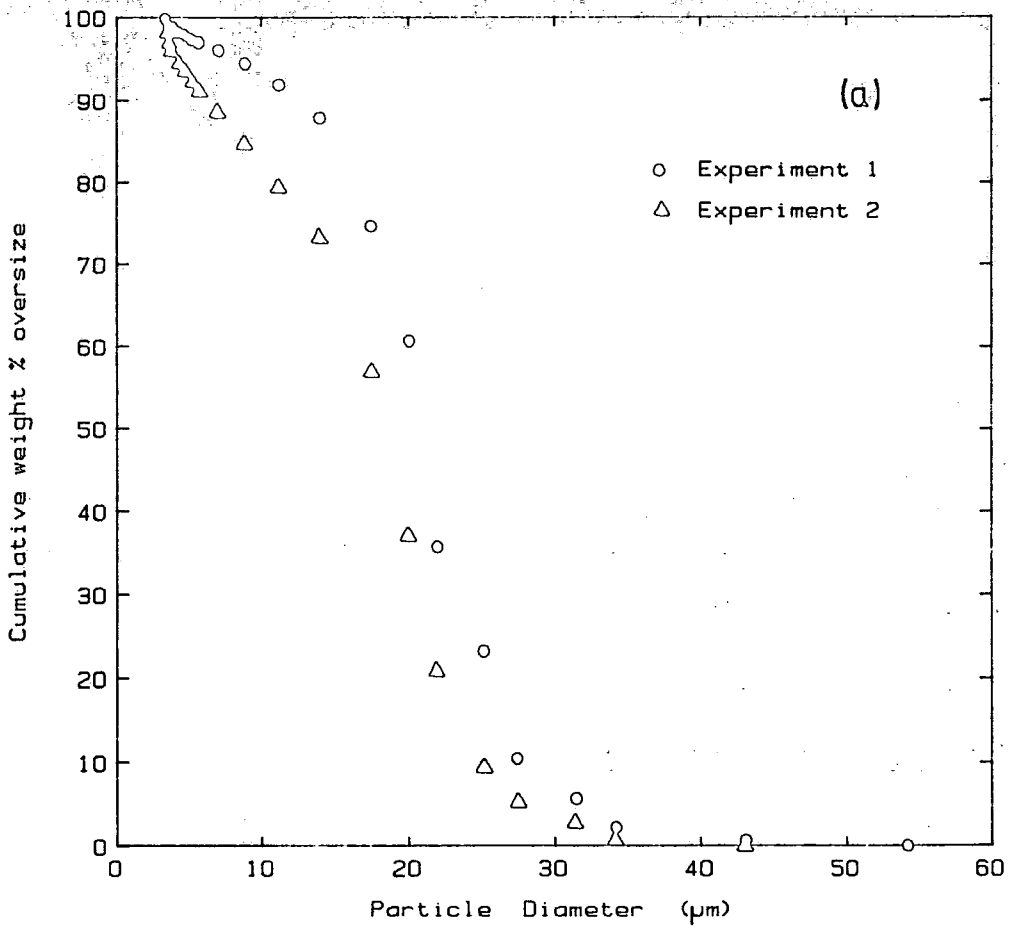
Coulter Counter size distribution study of HTC cells

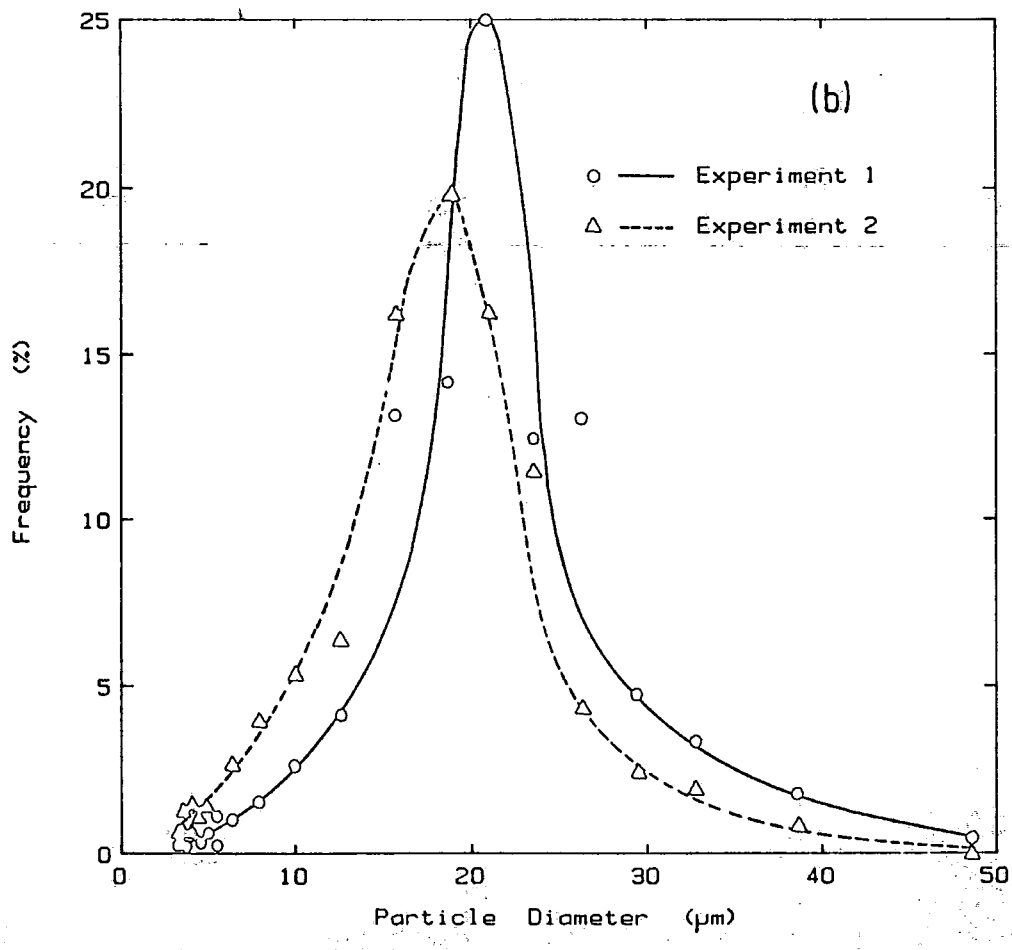
In two separate experiments, three $25cm^2$ flasks of exponentially growing cells were harvested with trypsin-EDTA solution and resuspended in $9ml$ of growth medium. A small volume ($2ml$) of this suspension was added to $250ml$ of Isoton II and a cell size distribution established with the Coulter Counter method outlined in Materials and Methods, section 2.2.3(c).

The data is represented in two forms:

(i) Cumulative frequency of cells of and above particular diameters (figure 2.2(a)).

(ii) Frequency of cells of particular diameters (figure 2.2(b)).


(a)
Experiment 1
Experiment 2
Cumulative weight % oversize
Particle Diameter (μm)
0
10
20
30
40
50
60
70
80
90
100


(b)
Experiment 1
Experiment 2
Frequency (%)
Particle Diameter (μm)
0
5
10
15
20
25
30
40
50

with diameters less than $6.97\mu m$, a finding reflected in the micrometer cell sizing studies.

Figure 2.2(b) shows the cell diameter frequency distribution established with the Coulter Counter. This figure confirms that the greatest frequency of cell diameter occurs in the 19.9 to $21.9\mu m$ range and that over 90% of cell diameters lie between 11.1 and $31.4\mu m$. Some difference between the two curves, obtained from two independent determinations, is to be expected since different samples of cells are likely to show variations in the proportions of cells present in the various stages of the cell cycle. From the graph it would appear that cells measured in run 1 contained more cells that were about to divide than was the case in run 2, since the frequency of larger cell diameters was greater in run 1 than in run 2.

Since the frequency of cells with diameters of less than $13.9\mu m$ was very low it was concluded that most HTC cells have diameters of $13.9\mu m$ or higher. On this basis $13.9\mu m$ was chosen as the minimum size of diameter to be used for measuring cells on the Coulter Counter. To measure diameters of this size required Coulter Counter settings of $A = 8$, $I = 0.017$ and $t = 20$.

To ensure that measurements made with the Coulter Counter at these settings were providing a true reflection of cell number a comparison was made between cells counted by haemocytometer and cells counted by Coulter Counter at the above settings.

The results in Table 2.1 indicate that the haemocytometer and Coulter Counter methods for counting cells produce highly comparable results when Coulter Counter settings that measure particles of $13.9\mu m$ and larger, are used. These Coulter Counter settings were therefore adopted in all future work.

2.3.2 Culture Techniques

(a) Flasks

Figure 2.3 shows the typical growth curves produced when varying numbers of cells were seeded into $25cm^2$ flasks. All three curves are characterised by an initial lag period of between 0 and 50 hours where there is little alteration in cell number. The length of this initial lag phase would appear to last longer when

Table 2.1

Comparison of methods for determining cell number

Three $25cm^2$ flasks of exponentially growing cells were harvested using a Trypsin-EDTA solution and the cells resuspended in growth medium. Cell counts were performed by haemocytometer and Coulter Counter at threshold, aperture and attenuation settings determined by earlier size distribution studies. Five $25cm^2$ flasks were each seeded with 200 cells in a total of $10ml$ of growth medium for each method of counting. Flasks were maintained in an incubator at $37°C$ under a humid atmosphere of air/CO_2 (19 : 1, v/v). The number of cells present in flasks was determined by the clonogenic assay outlined in Materials and Methods, section 2.2.3(d).

Haemocytometer number of colonies	Coulter Counter number of colonies
186	162
145	167
161	164
142	185
167	136

The difference between the Coulter Counter mean (162.8) and Haemocytometer mean (160.2) was tested for significance using the Student's t-test. The difference was not significant ($p > 0.05$).

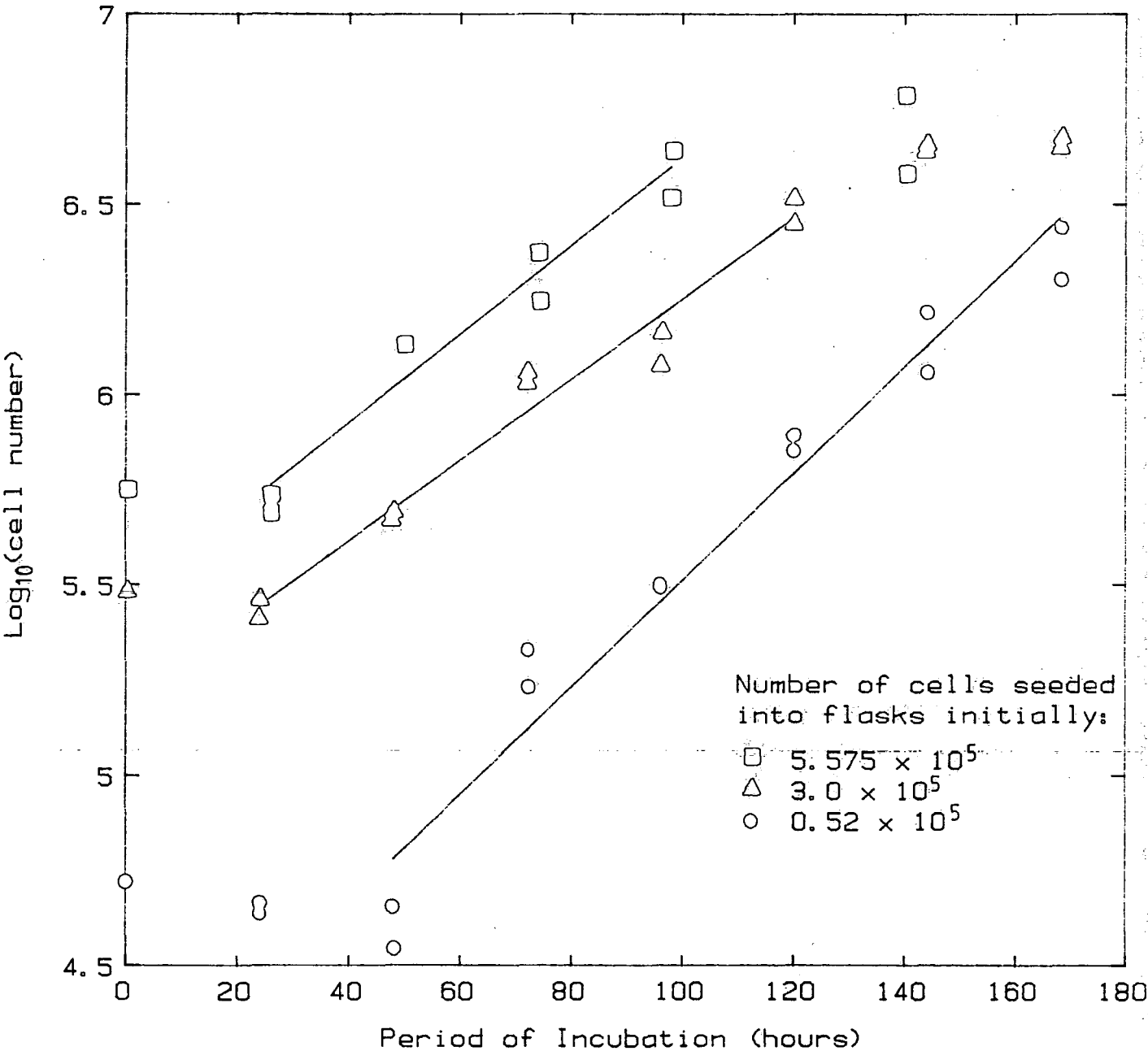
Figure 2.3

Growth characterisation of HTC cells maintained in $25cm^2$ flasks

On day 0 varying numbers of cells (5.2×10^4 to 5.575×10^5 per flask) were seeded into a number of flasks in a total of $10ml$ of growth medium. The flasks were maintained in an incubator at $37°C$ under a humid atmosphere of air/CO_2 ($19:1$, v/v). The medium was changed in flasks on day 1 and every 2 days thereafter. Growth was monitored at various times over the next 168 hours, by harvesting flasks in duplicate with trypsin-EDTA solution and counting the cells by Coulter Counter.

The three growth curves shown in the figure were derived from single experiments involving duplicate flasks.

Best fit regression lines were fitted to points in the exponential phase of growth using the regression equation $y = a + bx$.


7
6.5
6
5.5
5
4.5
0
20
40
60
80
100
120
140
160
180
Log10(cell number)
Period of Incubation (hours)
Number of cells seeded into flasks initially:
5.575 x 10^5
3.0 x 10^5
0.52 x 10^5

fewer cells are present, perhaps reflecting a lack of cell to cell contact. The cells then enter a period of rapid cell growth with a cell doubling time of approximately 24 to 26 hours. The rate of growth can be seen to be comparable under the different conditions of seeding, whilst the length of this exponential phase of growth shows some variation. When 5.575×10^5 cells were seeded into a $25cm^2$ flask the cells remained in the exponential phase of growth for approximately 70 hours. However, as the number of cells decreased, so the length of the exponential phase increased. It can be seen that when 5.2×10^4 cells were seeded into a flask they took longer to enter the exponential phase of growth and were still actively growing after 168 hours incubation. The major factors determining the length of the exponential phase include the surface area available for growth and the nutritional factors within the system. From the results it would seem that once the cells have covered the available surface area they enter a stationary phase. This suggestion is borne out by the fact that the two growth curves produced using the higher initial cell densities reach a plateau phase at approximately the same number of cells, despite regular media changes. From the graph it can be seen that this plateau phase occurs after approximately 140 hours incubation.

To ensure that cells used in subsequent experiments were all in the same exponential phase of growth, flasks were routinely seeded with 3×10^5 cells and then harvested 120 hours later for experimental purposes.

Table 2.2 shows the plating efficiencies obtained for HTC cells in the early stages of the study. The average plating efficiency was approximately 52%. It can be seen that cells seeded at different densities in flasks produce relatively constant plating efficiencies. Since there was some evidence of colonies merging together when higher numbers of cells were seeded into flasks, a cell density of 200 cells/flask was used as the seeding level in the majority of subsequent experiments.

Figure 2.4 shows the rate of cell attachment to substratum which was an important criterion to establish for conducting future experiments. It can be seen that after two hours there is no significant change in the percentage of cells attached to the flask. On the basis of these results, flasks that were seeded with

Table 2.2

Plating efficiency of HTC cells

A small number of cells (100 to 800 per flask) were seeded into a number of flasks on day 0 in $10ml$ growth medium. The flasks were maintained in an incubator at $37°C$ under a humid atmosphere of air/CO_2 (19 : 1, v/v). The medium was changed in flasks on day 1 and every two days thereafter. Colonies were fixed and counted on day 9 as described in Materials and Methods, section 2.2.3(d).

Number of cells seeded into flasks initially	Number of colonies counted	Plating efficiency* %
100	62.4 ± 3.67 (7)	62.4 ± 3.67
200	106.7 ± 5.47 (10)	53.4 ± 2.74
300	139.66 ± 5.78 (6)	46.6 ± 1.93
400	196.08 ± 5.13 (12)	49.0 ± 1.28
500	277.5 ± 14.5 (2)	55.5 ± 2.9
600	285.5 ± 10.5 (2)	47.6 ± 1.75
800	412.5 ± 16.0 (2)	51.6 ± 2.0

Numbers in brackets are numbers of replicates

Results represent mean value $\pm$ $S.E.M.$

$$* \ Plating\ Efficiency = \frac{Number\ of\ colonies\ counted}{Number\ of\ cells\ seeded\ initially} \times 100 \ .$$

Average plating efficiency = 52.3%

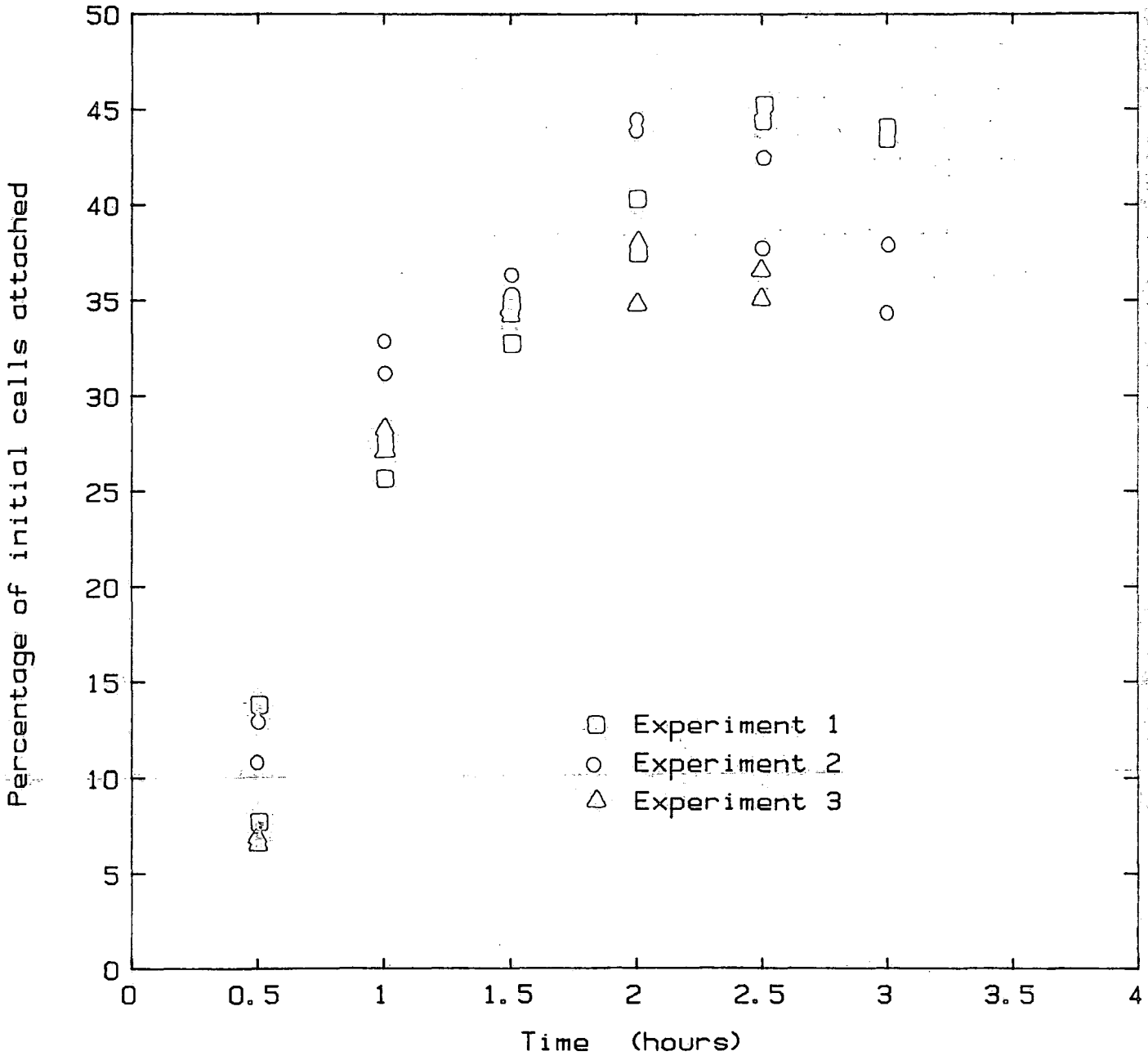
The plating efficiency values were analysed statistically for differences following arc sign transformation. There was no apparent statistical difference between plating efficiencies.

Figure 2.4

Rate of HTC cell attachment to substratum

Flasks ($25cm^2$) were seeded with 2.5×10^5 cells/flask in a total of $10ml$ growth medium and maintained in an incubator at $37°C$ under a humid atmosphere of air/CO_2 (19 : 1, v/v). Duplicate flasks were harvested every 30 minutes over the next three hours. Before harvesting, the medium containing unattached cells was discarded. Then the attached cells in each flask were harvested using Trypsin-EDTA solution and the cells were counted by Coulter Counter.

Individual results of three separate experiments run in duplicate are shown on the graph.


Percentage of initial cells attached
50
45
40
35
30
25
20
15
10
5
0
0
0.5
1
1.5
2
2.5
3
3.5
4
Time (hours)
Experiment 1
Experiment 2
Experiment 3

cells were always incubated for a minimum of two hours before being used in experimental work to ensure that cells were attached to the substratum.

(b) Plates

MTT assay

In the original Mosmann procedure (1983), $0.01ml$ MTT ($5mg/ml$ in phosphate buffered saline) was added to cells contained in $0.1ml$ culture medium (containing serum and phenol red indicator) in individual wells of a 96 well plate to produce insoluble formazan. After 4 hours at $37°C$ for MTT cleavage the dark blue formazan product was solubilised by adding an equal volume of isopropanol. Acid was added to the isopropanol to convert phenol red to its yellow form since the red form of this indicator was known to interfere with subsequent readings. The absorbance was then read at $570nm$ against a calibration wavelength of $630nm$.

In preliminary investigations modifications were made to this assay to enhance its suitability to the current study. Since the assay was to be run in 24 well plates as opposed to 96 well plates, optimum assay conditions needed to be redetermined. Initially, since precipitation of serum proteins was a major problem in the presence of the acid isopropanol, the serum containing medium was removed after MTT incubation prior to the addition of the acid isopropanol. However, removal of the media in this way often resulted in the loss of formazan product since cells frequently detached from wells during the MTT incubation period, particularly if the assay was run following exposure of the plates to elevated temperatures. In addition, the acid isopropanol often failed to solubilise the formazan adequately.

To improve the solubilisation of the formazan, dimethylsulphoxide (DMSO) was substituted for isopropanol following Alley et al. (1986) and Carmichael et al. (1987). In the presence of acid however, DMSO was found to produce much lower absorbance readings than it did when present on its own, as shown in Table 2.3.

To maintain the sensitivity of the assay a method was required in which acid could be excluded from the assay. In addition, to prevent the loss of formazan

Table 2.3

Influence of solvent composition on absorbance values in the MTT assay

Exponentially growing HTC cells were harvested with Trypsin-EDTA solution and two 24-well plates were seeded with varying numbers of cells (2.0×10^4 to 3.2×10^5 cells per well) in $1ml$ growth medium. The plates were maintained in an incubator at $37°C$ under a humid atmosphere of air/CO_2 (19 : 1, v/v). After 12 hours the medium was removed and one plate was assayed according to the final assay procedure given in Materials and Methods, section 2.2.4(b). The second plate was assayed in a similar fashion but with acid ($0.35ml$ concentrated HCl per $100ml$ DMSO) present in the DMSO.

Number of cells seeded into well	Absorbance at $540nm$	
	DMSO	Acid-DMSO
2.0×10^4	0.120 ± 0.004	0.068 ± 0.003
4.0×10^4	0.210 ± 0.003	0.122 ± 0.004
8.0×10^4	0.410 ± 0.013	0.236 ± 0.010
1.6×10^5	0.802 ± 0.024	0.429 ± 0.018
3.2×10^5	1.254 ± 0.029	0.864 ± 0.022

Numbers of replicates = 4

Results represent mean value $\pm$ $S.E.M.$

a method was needed that did not necessitate the removal of media after the addition of MTT. The preparation of medium in the absence of phenol red indicator and serum (Denizot and Lang, 1986) provided the solution. Fungizone was also omitted from the incubation medium in case of interference problems, whilst $10mM$ Hepes was included to help maintain a constant pH value in the small volume of incubation medium used in the assay. Hence, whilst cells were grown in normal serum-containing medium this was replaced with serum-free, phenol red-free, fungizone-free medium containing $10mM$ Hepes and MTT ($2.5mg/ml$) in the MTT assay.

Figure 2.5 shows the absorption spectrum of the formazan product. Since maximum absorption occurs around $540nm$ this was chosen as the test wavelength. The off-scale peak to the left of the formazan absorption peak represents the absorption produced by the unconverted MTT present in the incubation medium. Since the formazan clearly absorbs at $630nm$, a fact also shown by Denizot and Lang (1986), an alternative reference wavelength of $690nm$ was used at which neither formazan, nor MTT absorb.

Figure 2.6 shows the effect of using increasing MTT concentrations with constant cell number on the amount of formazan produced. It can be seen that the amount produced increases very rapidly from 0 to $1mg/ml$, slows between 1 and $2mg/ml$, plateaus between 2 and $3mg/ml$ and shows a decline above $3mg/ml$. At the higher concentrations the MTT is likely to be exerting a toxic effect on the cells. On the basis of these results $2.5mg/ml$ was selected as the optimal MTT concentration for use in future studies. Since cells were exposed to $200\mu l$ of $2.5mg/ml$ MTT per well, the amount of MTT present per well represents a tenfold increase on that used in Mosmann's original assay reflecting the larger scale of this procedure.

Figure 2.7 shows the effect of increasing the incubation period on the absorbance value obtained using a constant cell number with the optimal concentration of MTT. It can be seen that absorbance increases very markedly up to two hours and then declines slightly before reaching a constant value over the next 3 hours. In future studies, because the absorbance remained constant over the three to five hour range, plates were read during this time to ensure repro-

Figure 2.5

Absorption spectrum of formazan and MTT produced by the colorimetric assay

On day 0 a plate was set up containing 5×10^5 cells in $1ml$ of growth medium in two wells. The plate was maintained in an incubator at $37°C$ under a humid atmosphere of air/CO_2 (19 : 1, v/v). After 24 hours the following conditions were established:

(a) One well containing cells was exposed to $200\mu l$ of phenol red free, serum free, fungizone free medium (incubation medium) containing $2.5mg/ml$ MTT.

(b) One well containing cells was exposed to $200\mu l$ of incubation medium in the absence of MTT.

(c) One well without cells was exposed to $200\mu l$ of incubation medium containing $2.5mg/ml$ MTT.

The plate was incubated for 4 hours and then $1ml$ DMSO was added to each test well. A $1ml$ sample was removed from each well and an absorption spectrum determined on a Pye Unicam SP8-100 spectrophotometer after blanking the cuvette first on air.

The figure shows the absorption spectrum produced after exposure of cells to incubation medium containing $2.5mg/ml$ MTT. Analysis of absorption spectra produced by DMSO alone and also by conditions (a), (b) and (c) above has shown that the peak on the right hand side represents the absorption spectrum for formazan with an absorption maximum around $540nm$, whilst the off-scale peak represents the absorption peak of unconverted MTT present in the incubation medium at the end of the 4 hour incubation period.

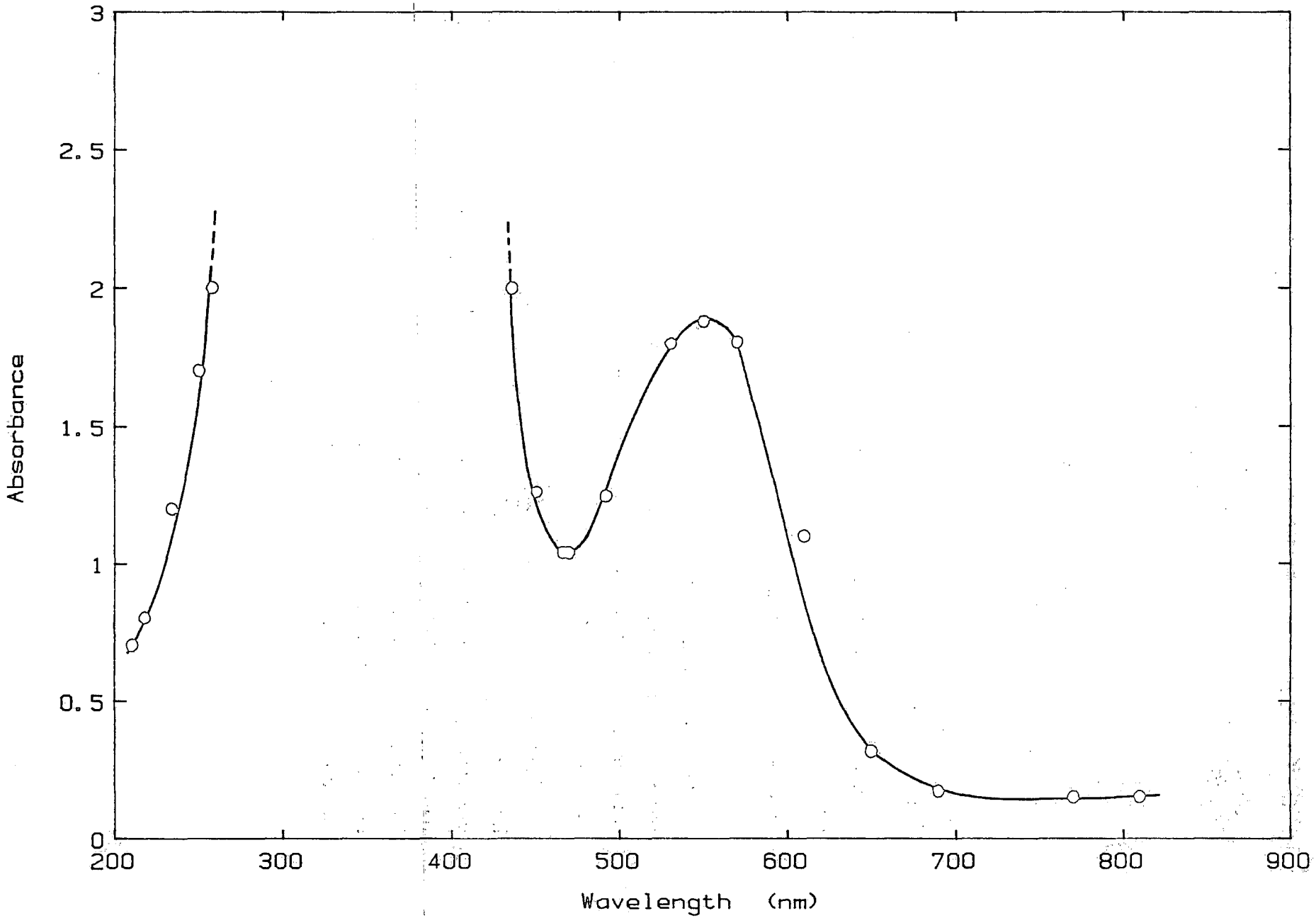

Absorbance
3
2.5
2
1.5
1
0.5
0
200
300
400
500
600
700
800
900
Wavelength (nm)

Figure 2.6

Effect of MTT concentration on absorbance

Exponentially growing HTC cells were harvested using trypsin-EDTA solution and one 24-well plate was seeded with 1.0×10^5 cells per well in $1ml$ growth medium. The plate was maintained in an incubator at $37°C$ under a humid atmosphere of air/CO_2 (19 : 1, v/v). The medium was changed 24 hours later. After a total of 36 hours the plate was exposed to varying concentrations of MTT (0.5 to $4.0mg/ml$) for a 4 hour period at $37°C$ and processed as described in Materials and Methods, section 2.2.4(b).

Each point represents the mean $\pm S.E.M.$ of 4 replicate wells.

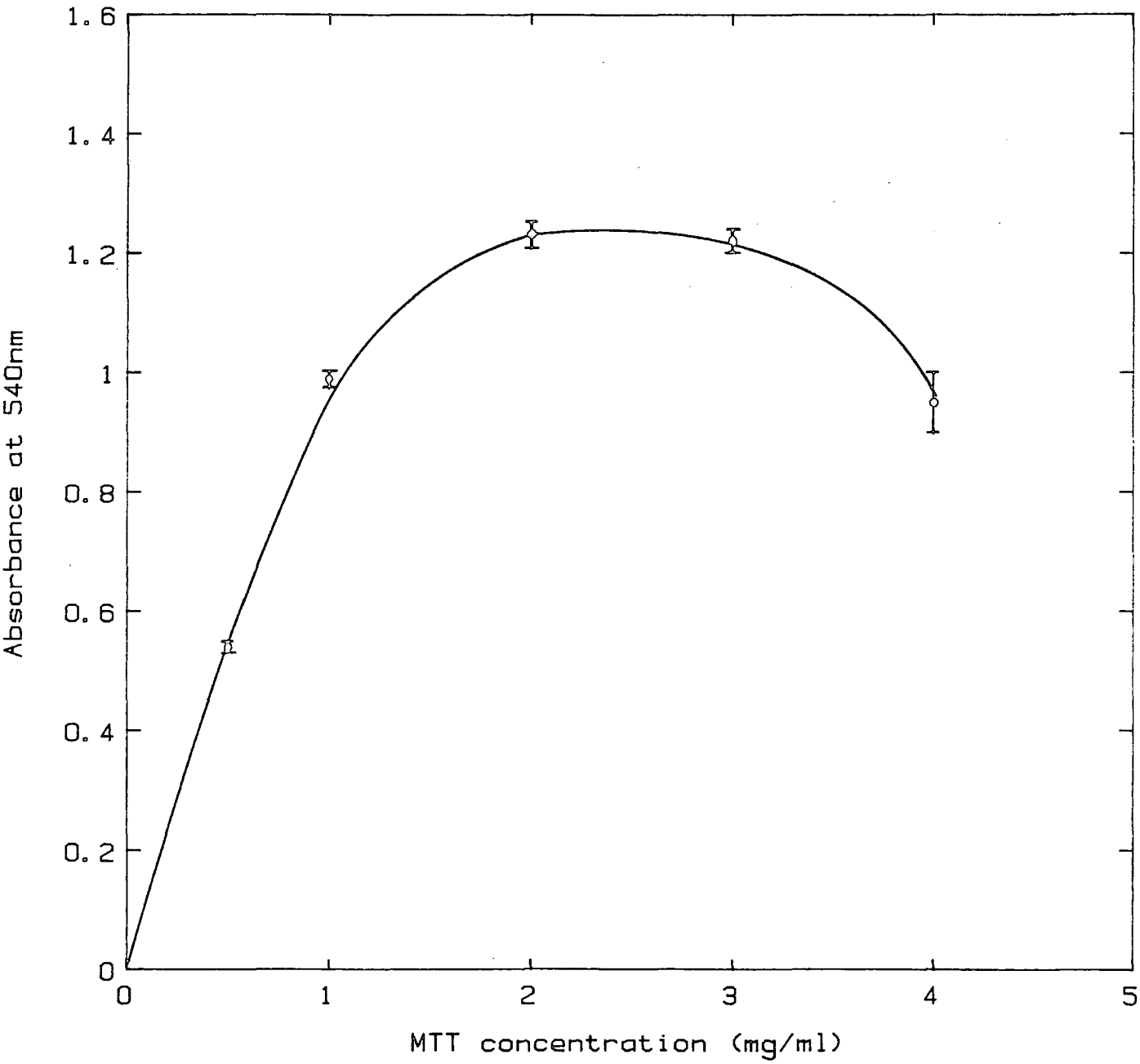

1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Absorbance at 540nm
0
1
2
3
4
5
MTT concentration (mg/ml)

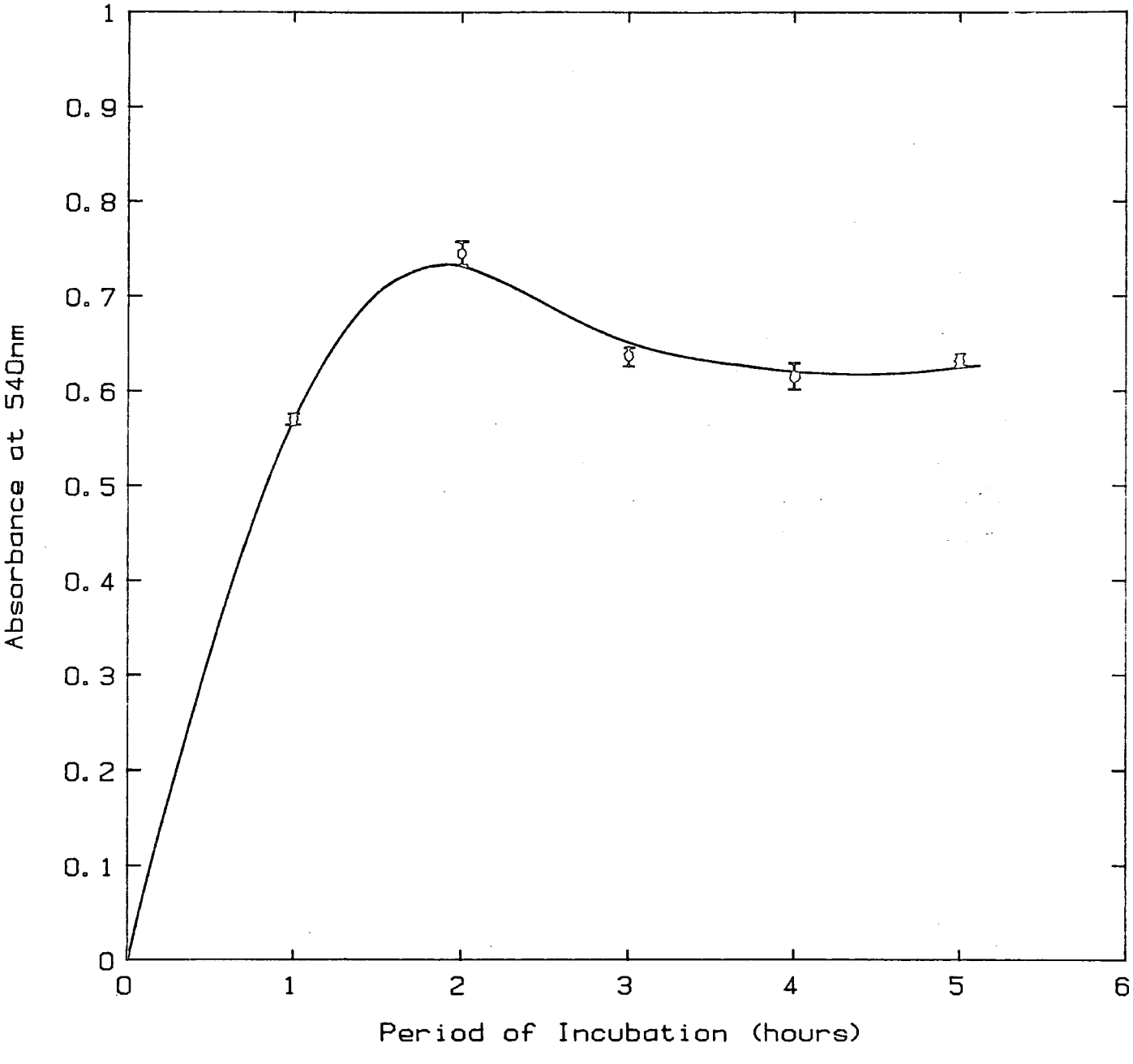
Figure 2.7

Effect of MTT incubation period on absorbance

Exponentially growing HTC cells were harvested using trypsin-EDTA solution and 24-well plates were seeded with 5.0×10^4 cells per well in $1ml$ growth medium. Plates were maintained in an incubator at $37°C$ under a humid atmosphere of air/CO_2 (19 : 1, v/v). The medium was changed 24 hours later. After a total of 36 hours, plates were exposed to $2.5mg/ml$ MTT at $37°C$ for varying time periods (1 to 5 hours) and processed as described in Materials and Methods, section 2.2.4(b).

Each point represents the mean $\pm S.E.M.$ of 8 replicate wells.

The absorbance value after 2 hours incubation was significantly different ($p < 0.001$) from that obtained after 3 hours. However, since absorbance values obtained at 3, 4 and 5 hours incubation were not significantly different ($p > 0.1$ in both cases), a period of 3-5 hours was chosen as the optimal incubation period. This incubation period was chosen to ensure that reproducible absorbance values would be obtained even if the incubation period altered slightly.


Absorbance at 540nm
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
1
2
3
4
5
6
Period of Incubation (hours)

ducible results were obtained in the event of slight differences in the length of the incubation period.

Obviously for this modified version of Mosmann's colorimetric assay to be of use in the current study the absorbance values generated must have a linear relationship with cell number. Figure 2.8 shows the effect of increasing the cell number (from 0 to 5.2×10^5 cells/well) on the absorbance value generated through formazan production, under the optimal conditions of MTT concentration ($2.5mg/ml$) and incubation period (4 hours) established for the assay. It can be seen that a linear relationship ($r = 0.990$) exists between cell number and absorbance for up to 2.1×10^5 cells per well. Since each well only has a $2.01cm^2$ area available for growth there is a limit to the number of cells that can be introduced into a well without overcrowding it. The graph would suggest that well cell number should not exceed 2.1×10^5, nor absorbance 0.900 absorbance units, if reliable results are to be obtained with this assay. Thus the results shown in figure 2.6 are less than ideal since a number of the points exceed the 0.900 limit. However the general trend reflected in this figure is in keeping with other workers (Denizot and Lang, 1986) which is why it has been included in the present results. On the basis of the cell number-absorbance relationship, future plate experiments were designed to ensure, as far as possible, that final experimental absorbance values were below 0.900 absorbance units so that the absorbance value accurately reflected the number of cells in the well. This in turn enabled estimates of cell numbers to be made from the absorbance values, so that comparisons could be drawn with cell determinations made in the alternative flask experimental systems.

Figure 2.9 shows the absorbance values produced by incubating various numbers of cells (4 to 8×10^3 per well) over a seven day period in 24-well plates under standard assay conditions. After one day the absorbance values reflect the original number of cells seeded into the plates suggesting cells are experiencing a lag phase in their growth. After two days the absorbance has doubled, and since this reflects the amount of formazan produced which is proportional to cell number, it can be assumed that cell number has also doubled indicating that the cells have entered the active phase of growth. Once again, as with the flasks, the length of this active period of growth can be seen to vary. Theoretically the

Figure 2.8

Relationship between cell number and absorbance reading obtained in plates

Exponentially growing HTC cells were harvested using trypsin-EDTA solution and 24-well plates were seeded with varying numbers of cells (0 to 5.2×10^5 cells/well) in $1ml$ growth medium. Plates were maintained in an incubator at $37°C$ under a humid atmosphere of air/CO_2 (19 : 1, v/v). After 12 hours plates were exposed to $2.5mg/ml$ MTT at $37°C$ for a 4 hour period and processed as described in Materials and Methods, section 2.2.4(b).

The results from three separate experiments are shown. Each point represents the mean value derived from 4 replicate wells.

The best fit regression line has been fitted to the linear region of the graph using the regression equation $y = a + bx$.

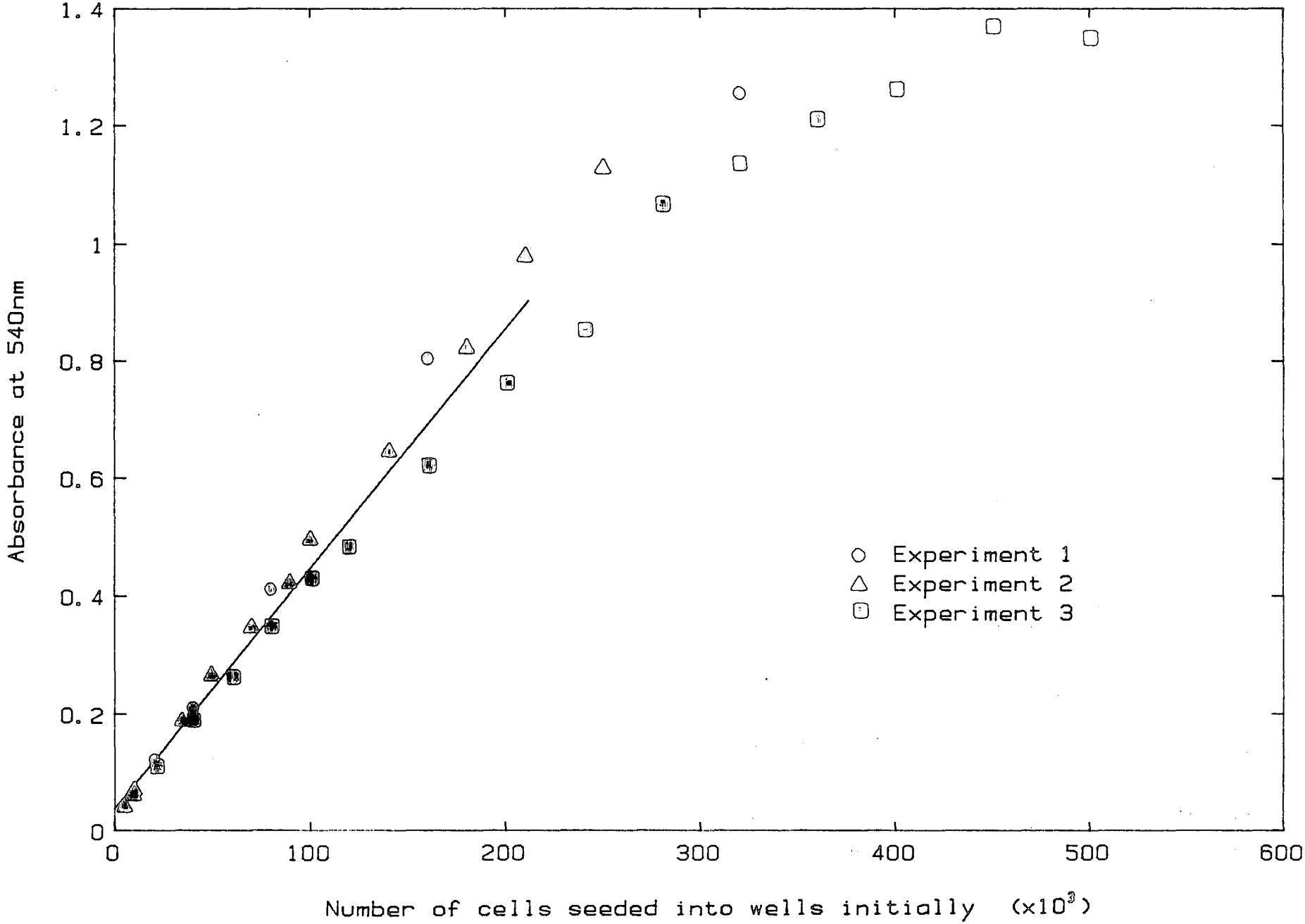
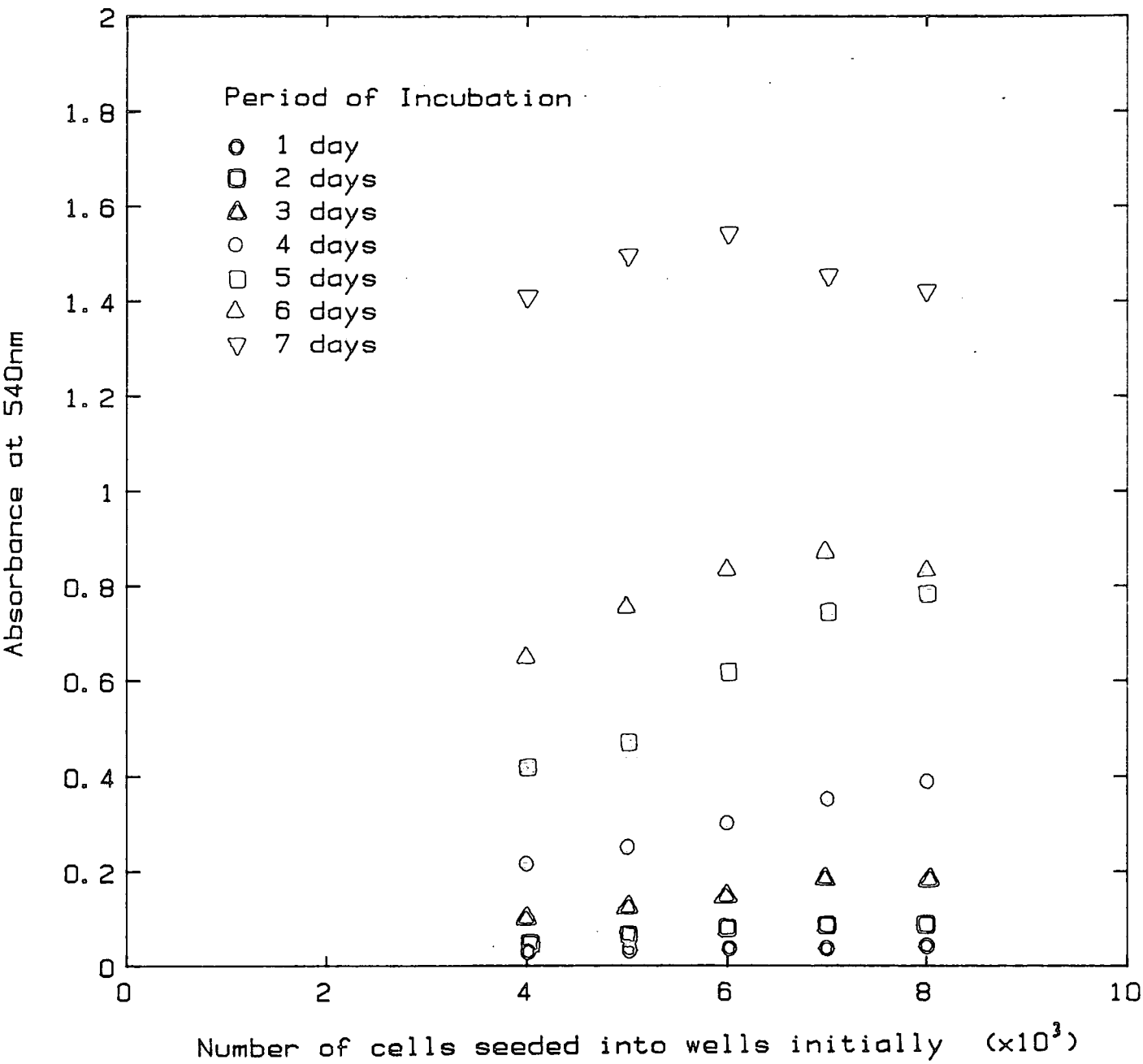

1.4
1.2
1
0.8
0.6
0.4
0.2
0
Absorbance at 540nm
0
100
200
300
400
500
600
Number of cells seeded into wells initially (x10³)
Experiment 1
Experiment 2
Experiment 3

Figure 2.9

Influence of cell number on absorbance readings obtained in 24-well plates

On day 0 exponentially growing HTC cells were harvested using trypsin-EDTA solution and seven 24-well plates were seeded with varying numbers of cells (4 to 8×10^3 cells/well) in $1ml$ growth medium. The plates were maintained in an incubator at $37°C$ under a humid atmosphere of air/CO_2 (19 : 1, v/v). The medium was changed in appropriate plates on days 1, 3 and 5. After 24 hours (day 1) one plate was exposed to $2.5mg/ml$ MTT at $37°C$ for a 4 hour period and processed as described in Materials and Methods, section 2.2.4(b). This procedure was repeated every 24 hours over the next six days.

The results from a single experiment, run over 7 days are shown. Each point represents the mean value derived from 4 replicate wells.


Period of Incubation
1 day
2 days
3 days
4 days
5 days
6 days
7 days
Absorbance at 540nm
Number of cells seeded into wells initially (x10³)
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
1.8
2
0
2
4
6
8
10

absorbance values obtained with 8×10^3 cells per well should be more or less double that obtained with 4×10^3 cells after equivalent periods of incubation. Although this is the case after two to four days incubation, by day 7 it can be seen that these two absorbance values are very similar which suggests that cells in both wells have reached confluency and have entered a stationary phase of growth. Since the absorbance values obtained on day 7 exceed the maximum 0.900 level permissible for cell number-absorbance linearity it is difficult to know how accurately these absorbance values reflect cell number.

In Table 2.4 absorbance values from the data shown in figure 2.9 have been converted into approximate cell numbers on the basis of the linear regression line determined for the three sets of data shown in figure 2.8. Since the linear regression line does not pass through the origin, estimates of cell number based on very low absorbance values are difficult to obtain. As the absorbance values increase so it becomes easier to estimate the likely cell number. Taken as a whole, the estimated cell number as would be expected, reflects the lag, log and stationary phases of growth suggested by the absorbance values.

Figure 2.10 shows a selection of growth curves produced in plates based on cell number estimates. Allowing for the fact that it is difficult to estimate cell number reliably at low absorbance values, it can be seen that the growth curves produced in plates are highly comparable to those produced in flasks. The gradients of the slopes during the exponential phase of cell growth are very similar indicating comparable doubling times. The fact that cells showed similar growth characteristics in plates and flasks enabled both types of vessel to be used interchangeably in subsequent experiments.

On the basis of the data presented in this chapter whenever flasks were to be used for experimental purposes they were seeded with 200 cells/flask. The cells for these flasks were obtained from 'stock' flasks containing exponentially growing cells which was achieved by seeding $25cm^2$ flasks with 3×10^5 cells and incubating the stock flasks for a five day period prior to use (figure 2.3). After seeding, experimental flasks were left for a minimum of two hours in the incubator (figure 2.4) to allow attachment of the cells to the flask. The appropriate experiment

Table 2.4

Growth characteristics of cells maintained in 24-well plates

Plates (24-well) were seeded with varying numbers of cells (4×10^3 to 8×10^3 per well) on day 0 and treated as described in the legend of figure 2.9.

The results given in the table were derived from a single experiment run over 7 days. Estimated cell numbers have been determined from figure 2.8.

Period of incubation (Days)	Absorbance (ABS) at 540nm and estimated cell number (ECN) in wells seeded with varying cell number on day 0									
	4×10^3 cells		5×10^3 cells		6×10^3 cells		7×10^3 cells		8×10^3 cells	
	ABS	ECN	ABS	ECN	ABS	ECN	ABS	ECN	ABS	ECN
1	0.027 ±0.002	1.0×10^3	0.031 ±0.002	1.2×10^3	0.044 ±0.002	4.5×10^3	0.034 ±0.002	2.5×10^3	0.042 ±0.002	4.0×10^3
2	0.048 ±0.001	5.0×10^3	0.061 ±0.001	8.0×10^3	0.071 ±0.002	1.1×10^4	0.075 ±0.001	1.15×10^4	0.085 ±0.001	1.4×10^4
3	0.098 ±0.002	1.7×10^4	0.129 ±0.003	2.4×10^4	0.152 ±0.002	3.0×10^4	0.177 ±0.004	3.5×10^4	0.183 ±0.001	3.7×10^4
4	0.214 ±0.003	4.45×10^4	0.249 ±0.004	5.15×10^4	0.298 ±0.002	6.3×10^4	0.352 ±0.004	7.65×10^4	0.387 ±0.008	8.4×10^4
5	0.415 ±0.010	9.05×10^4	0.469 ±0.010	1.03×10^5	0.620 ±0.007	1.38×10^5	0.742 ±0.007	1.67×10^5	0.785 ±0.010	1.78×10^5
6	0.647 ±0.010	1.44×10^5	0.756 ±0.016	1.71×10^5	0.834 ±0.008	1.89×10^5	0.871 ±0.013	1.98×10^5	0.830 ±0.007	1.88×10^5
7	1.410 ±0.013	–	1.496 ±0.025	–	1.542 ±0.012	–	1.455 ±0.011	–	1.423 ±0.022	–

Numbers of replicates = 4

Results represent mean value ± $S.E.M.$

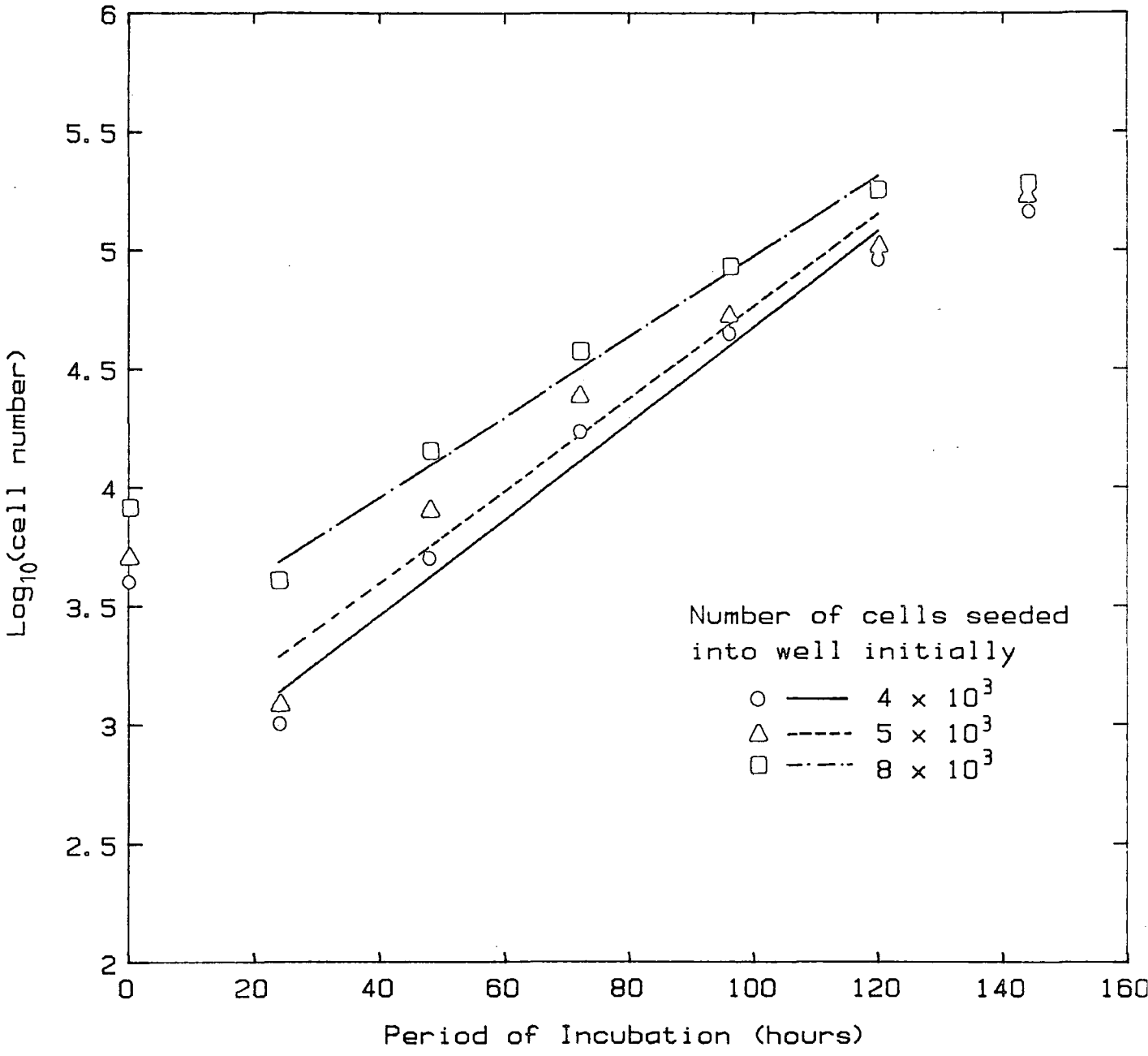
Figure 2.10

Growth curves of cells in plates estimated using MTT assay

On day 0 varying numbers of cells (4 to 8 $\times$ 10^3 cells/well) were seeded into a number of 24-well plates in a total of $1ml$ growth medium. The plates were maintained in an incubator at $37°C$ under a humid atmosphere of air/CO_2 (19 : 1, v/v). The medium was changed in wells on day 1 and every 2 days thereafter. Growth was monitored at various times over the next 144 hours using the modified Mosmann colorimetric assay described in Materials and Methods, section 2.2.4(b).

The three growth curves shown were derived from single experiments. Each point represents the mean value derived from 4 replicate wells.

Best fit regression lines have been fitted to the points in the exponential phase of growth using the regression equation $y = a + bx$. The gradients of the 3 slopes are not significantly different.


6
5.5
5
4.5
4
3.5
3
2.5
2
0
20
40
60
80
100
120
140
160
Log10(cell number)
Period of Incubation (hours)
Number of cells seeded
into well initially
4 x 10^3
5 x 10^3
8 x 10^3

would then be conducted after which time the flasks would be returned to the incubator for a further nine days before the colonies were counted.

Alternatively when plates were used for experimental purposes, to enable them to be run over a 6 day period without the cells reaching confluency or the absorbance exceeding 0.900 they were seeded with 5×10^3 cells per well on day 0 (figure 2.9) from exponentially growing flasks of cells. Experiments were conducted on day 3 when the cells were in the exponential phase of growth (figure 2.10) and subjected to the MTT assay on day 6.

(c) Techne flasks

In preliminary studies two of the microcarriers available, namely Cytodex 2 and Cytodex 3, were tested to determine which one was more suited to the growth of HTC cells. Since cells proved more difficult to detach from Cytodex 3, use was made of Cytodex 2 in the current study.

Figure 2.11(a) shows a scanning electron micrograph of HTC cells growing on a Cytodex 2 microcarrier bead 72 hours after inoculation. It can be seen that the cells form a monolayer over the microcarrier bead surface. Boundaries of individual cells are apparent and the cell surfaces are characterised by the presence of a large number of microvilli. Figure 2.11(b) shows a low power transmission electron micrograph of an HTC cell attached to a Cytodex 2 microcarrier bead. The cell can be seen to be closely associated with the bead surface and at the subcellular level it is characterised by a fairly large nucleus and a number of mitochondria.

The yield of cells produced from the microcarrier cultures were rather lower than expected. Using the optimal concentration of microcarrier of $3mg/ml$ final volume and an inoculation density of 8×10^4 cells/ml suggested by Pharmacia (Pharmacia Fine Chemicals, 1981) the surface area provided by the microcarrier ($5500cm^2/g$ dry Cytodex 2) should have ensured a cell yield of approximately 5.5×10^5 cells/mg Cytodex. However, seeding four Techne flasks, each containing $1.5g$ of Cytodex 2, with 40 million cells per flask typically yielded 8×10^8 cells in total following 4 to 6 days growth, i.e. approximately one quarter of the yield expected.

Figure 2.11

Scanning and Transmission electron micrographs of HTC cells grown on Cytodex 2

HTC cells were cultured on Cytodex 2 microcarrier beads in a Techne flask for 72 hours and prepared for scanning and transmission electron microscopy as described in Materials and Methods, section 2.2.2(b).

(a) Scanning electron micrograph of HTC cells on a Cytodex 2 microcarrier bead. The bar represents $20\mu m$.

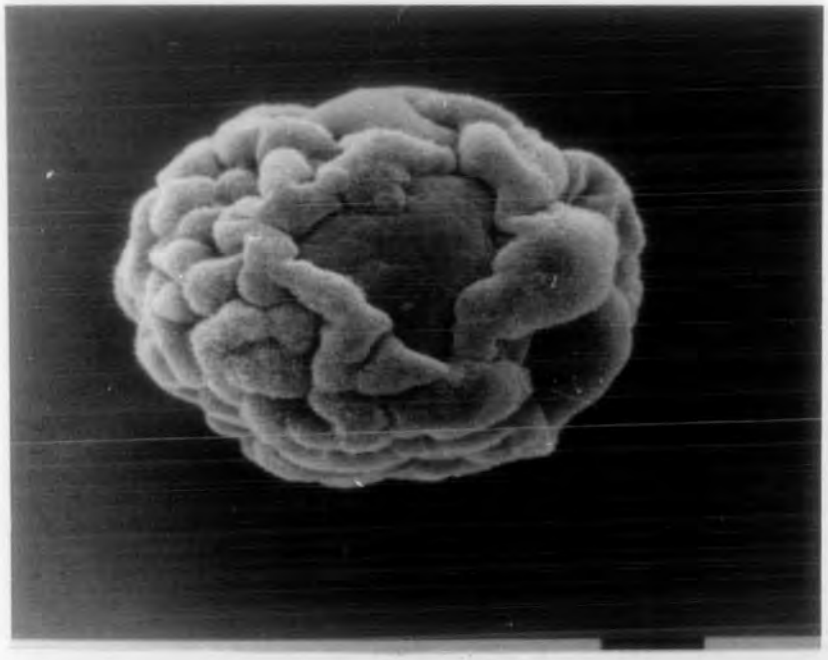
(b) Transmission electron micrograph ($\times 12000$) of an HTC cell attached to a Cytodex 2 microcarrier bead. Several features are identifiable:

N = nucleus

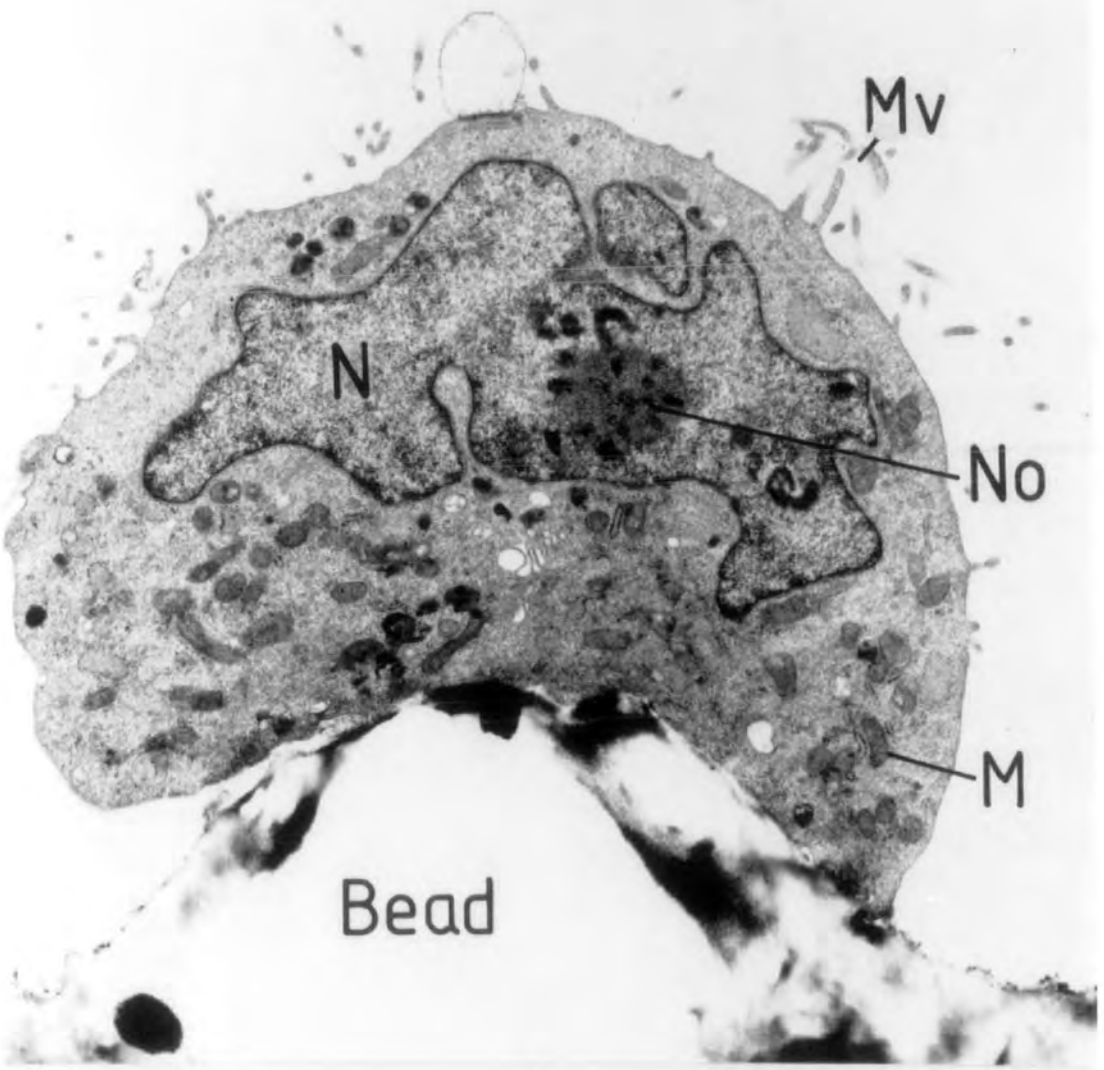
M = mitochondrion

N_0 = nucleolus

M_v = microvillus



a



b

(d) Cryopreservation

The composition of the freezing medium was varied in initial studies to ensure that HTC cells were stored in the most viable state. Four freezing media were compared namely (a) growth medium (containing 10% NBS)/glycerol (92 : 8, v/v), (b) Newborn bovine serum/glycerol (92 : 8, v/v), (c) Foetal bovine serum/glycerol (92 : 8, v/v), (d) Foetal bovine serum/DMSO (92 : 8, v/v). Newborn bovine serum (NBS) was included in the first two freezing media because the cells were grown routinely in medium containing 10% NBS. Cell survival was not determined quantitatively in these preliminary experiments, but visual observation revealed that the foetal bovine serum/glycerol and foetal bovine serum/DMSO mixtures gave the highest cell survivals and HTC cells have been frozen successfully at $-196°C$ in these media for up to 26 months.

2.4 Discussion

The present chapter has defined conditions for the successful growth of HTC cells in monolayer culture in multiwell plates, flasks or attached to microcarrier beads in suspension.

Characterisation of the growth patterns of this particular cell line was fundamental if suitable experimental procedures were to be designed for future work. The growth curves obtained for these cells in both plates and flasks compare favourably with that described by Thompson et al. (1966). In that study aliquots of cells were grown in petri dishes, and a doubling time during the logarithmic phase of growth of approximately 24 hours was reported. Schamhart et al. (1984) reported a doubling time of 22 hours. Thus the doubling time of approximately 24 to 26 hours determined under the present culture conditions reflects that of other workers.

The plating efficiency of these HTC cells, which is a measure of the cells' ability to survive the subculture process, showed some variation over the course of the study from approximately 40 to 80%. Thompson et al. (1966) reported a figure of 50 to 100%. Some variation in plating efficiency is to be expected since it is clearly dependent on the culture procedure and conditions. For example when enzymes are used to detach cells from the substrate slight variations in

enzyme concentration or the time of exposure of the enzyme to the cells can lead to increased cell surface damage, which will reduce cell viability and hence the plating efficiency that is recorded. The fact that enzymes, often when used in conjunction with chelating agents such as EDTA, can alter cell viability has been shown by a number of workers (Anghileri and Dermietzel, 1976). Similarly plating efficiencies are also much higher when cells in exponential growth are used rather than cells from a resting population (Pharmacia Fine Chemicals, 1981).

In the present study an increase in plating efficiencies was observed when a Coulter Counter was employed for cell counting purposes. The Coulter Counter provided a much more rapid method for counting cells than the alternative haemocytometer method with the result that cells were exposed to stressful subculture conditions for shorter periods of time, and this could account for higher plating efficiencies.

The modifications made to the original Mosmann method (1983) for assessing cell viability have provided a reliable, sensitive and rapid means of assessing cell number in 24 well plates, provided absorbance values do not exceed a specified value.

Mosmann (1983) reported a linearity between cell number and amount of formazan generated in 96 well plates for up to 5×10^4 cells/well using a mouse lymphoma cell line. Green et al. (1984), using Mosmann's method with human and mouse fibroblasts, again with 96 well plates, reported linearity up to 2×10^5 cells/well though at cell concentrations greater than 3×10^4/well the cultures were overcrowded and did not maintain an even monolayer configuration. In the present system where there is a larger surface area available for growth ($2.01 cm^2$ as opposed to $0.28 cm^2$ in 96 well plates) linearity exists for up to 2.1×10^5 cells/well. However, linearity in terms of number of cells per unit area are comparable in the 3 assay procedures. The study of Mosmann (1983) indicates linearity up to 1.7×10^4 cells/cm^2, and the study of Green et al. (1984) indicates linearity up to 1.07×10^4 cells/cm^2 whilst the current study indicates linearity up to 1.24×10^4 cells/cm^2.

The MTT concentration curve produced in this study reflects that of Denizot and Lang (1986), except at higher concentrations of MTT. Whilst Denizot

and Lang found that the amount of formazan produced began to reach a plateau above $2mg/ml$, a decline in formazan production was seen with higher concentrations of MTT in the current study. This difference is probably due to a toxicity effect of the MTT on the cells in this study since the amount of MTT exposed to cells was ten times higher than that employed by Denizot and Lang, even though the concentration was the same.

The optimal incubation period of 3 to 5 hours used with this assay has also been adopted by other workers. Mosmann (1983) selected a 4 hour incubation period whilst Denizot and Lang (1986) chose a shorter incubation period of 3 hours for their standard procedure to reduce the time of the assay procedure, since they found that there was a gradual increase in formazan production with time which levelled off after 3-4 hours.

An important point to bear in mind is that whilst this assay, developed for use with plates, measures the metabolic activity of the cells, the clonogenic assay that is used with flasks measures the reproductive ability of cells. Thus it is likely that different results will be obtained from the two assay procedures under similar experimental procedures, in which cells are exposed to heat stress, since a cell may lose its reproductive ability but still maintain a degree of metabolic activity. The choice of assay should depend on the nature of the information sought. Also, when plates are being used as the experimental system, care must be taken to ensure that all cells are in the exponential phase of growth at the time of the assay. If control cells from experiments are allowed to enter the stationary phase, whilst treated cells continue to grow, and results are interpreted on the basis of differences in absorbances between control and treated cells, serious inaccuracies may result.

The major reason for the rather lower than expected yields of cells from the microcarrier cultures appeared to be because not all the microcarrier beads supported cell growth. Clearly the key to achieving maximum cell yields from microcarrier cultures is to ensure that all microcarriers bear cells from the very beginning of the culture since transfer of cells from one microcarrier to another occurs fairly infrequently during the culture period as a result of constantly stirring the microcarrier suspension. Although cultures were initiated in reduced

volumes of medium to enhance the chances of cells coming into contact with all microcarriers it would seem that further measures are called for to increase cell yields. Some workers have found that variations in the inoculation density can improve cell yield. For example, Horng and McLimans (1975) reported that approximately 5 cells/microcarrier was the ideal inoculation level when using anterior calf pituitary cells to ensure maximum utilisation of the microcarriers. With human fibroblasts this number rises to approximately 10 cells/microcarrier (Pharmacia Fine Chemicals, 1981). Thus it could well be, that higher yields of HTC cells from microcarrier culture could be achieved in the future by increasing the inoculation density to a value above the 5 cells/microcarrier that is currently used. The number of cells attaching to microcarriers and hence cell yield will also be affected by culture conditions. For example since pH influences cell survival, attachment, growth and function, maintaining the correct pH is central to obtaining optimal cell growth. A decrease in pH is one of the most common causes of poor results in microcarrier culture and undoubtedly had an effect in the current study, since cultures were maintained in closed culture systems where pH was difficult to maintain at a constant level. Further studies involving microcarrier culture should pay even more attention to optimising conditions during all stages in the growth of the culture, since deviations at any stage usually have a dramatic effect on final cell yields.

The cryopreservation technique adopted in the current study involving the use of glycerol and foetal bovine serum has proved to be very successful for the storage of HTC cells in a viable state. Although DMSO was found to work equally well as cryoprotectant it was not employed due to reports of its potential for stimulating gene activation and differentiation effects in certain cell lines (Rudland et al., 1982). Successful freezing of HTC cells in growth medium/glycerol ($95:5$, v/v) has been reported by Thompson et al. (1966). However, the present attempt to develop an analogous method of freezing employing a different type of growth medium to that used by Thompson and a slightly higher level of glycerol (8% as opposed to 5%) did not prove useful in the current study and was not adopted.

Chapter III

Thermal Sensitivity of HTC cells and the Effects of Fatty Acid Supplementation

3.1 Introduction

Mammalian cells experience heat injury and heat death at temperatures only slightly above $40°C$. As a result the effect of hyperthermia at $41°C$-$46°C$ on cell viability has been studied extensively *in vivo* and *in vitro* because of its potential in the treatment of human cancers.

Whilst mammalian cells are known to suffer heat injury and heat death at temperatures only slightly above their normal range and whilst a number of cell culture studies have shown tumour cells to be more thermosensitive than their normal counterparts (Giovanella et al., 1976), the mechanism of cell death is not well understood. Part of the problem is that temperature has an all pervasive influence on cellular structures which makes it difficult to identify the primary sites of lesion. All the major cellular structures have been implicated as having a significant role in heat injury at one time or another (Roti Roti, 1982).

However, increasing attention is being focused on membranes as a site for hyperthermic damage to cells since heat has been shown to alter many properties of the plasma membrane. Changes have been reported in the permeability and transport of many compounds. For example, inhibition of Na^+-dependent amino acid transport has been observed (Lin et al., 1978), the active transport of Na^+ and K^+ appears to be inhibited and there is loss of K^+ (Burdon and Cutmore, 1982). The binding of growth factors and mitogens to plasma membranes (Magun and Fennie, 1981) and the surface morphology of plasma membranes (Bass et al., 1978; Borrelli et al., 1986) has been shown to be altered as a result of hyperthermic treatment.

Morphological evidence suggesting the importance of the plasma membrane as a site for hyperthermic damage comes from a number of sources. Fajardo et

al. (1980) observed the heat destruction of the plasma membrane in a tumour cell line, whilst a discontinuous plasma membrane has also been observed by Schrek et al. (1980) in heated lymphocytes. The loss of microvilli (Mulcahy et al., 1981) and the formation of blebs (Bass et al., 1978; Kapiszewska and Hopwood, 1986) are a common observation in heat damaged cells. Bass et al. (1982) demonstrated a good correlation between membrane blebbing and cell killing for hyperthermic exposures at $42°C$ and $43°C$. Similar work by Borrelli et al. (1986) has demonstrated a direct correlation between the degree of membrane blebbing and cell lethality when synchronous G_1 CHO cells are heated at $45.5°C$ for 3-20 minutes.

The significance of membranes as a possible target for cellular heat damage was emphasised by Bowler et al. (1973) from work on ectothermal animals. It has long been known that the heat death points of ectotherms are dependent on the acclimation history of the animals (see Cossins and Bowler, 1987). However, what is also evident is that during thermal acclimation ectotherms remodel their membrane lipids (Johnson and Roots, 1964; Cossins et al., 1977; Hazel, 1979). Cossins et al. (1977) have shown that changes occur in the 'fluidity' of goldfish brain synaptosomal membranes that parallel the changes in lipid composition. Similar relationships have been reported for Tetrahymena membranes by Martin and Thompson (1978). This remodelling of phospholipid fatty acid composition during thermal acclimation in ectotherms is believed to be an adaptive response, with the incorporation of unsaturated fatty acids in the cold resulting in an increase in disorder in the bilayer which serves to compensate for the direct ordering caused by the lower temperature. Sinensky (1974) has coined the term 'homeoviscous adaptation' to describe this phenomenon. One feature of the less ordered bilayer in the cold acclimated condition is that it is likely to suffer greater perturbation by high temperatures than the more ordered membranes from warm acclimated cells. Evidence for this comes from a number of studies. For example, Esser and Souza (1974) found a correlation between membrane fluidity and thermal death in Bacillus stearothermophilus whilst Cossins et al. (1981) showed that the Na^+/K^+ATPase in the synaptic membranes from warm acclimated goldfish was more thermally stable than the same enzyme in cold acclimated membranes (Bowler, 1987). This latter study in particular suggests

that the thermostability of membrane proteins is affected by the level of 'fluidity' of the bilayer.

The cells of most mammals do not experience changes in temperature associated with season and so may not possess the same range of compensatory adaptations observed in ectotherms. However, there is some evidence that Chinese hamster ovary cells in culture show a similar capacity to alter their thermal resistance depending on culture (growth) temperature (Culver and Gerner, 1982) although it is not clear whether corresponding changes in membrane composition and physical state occur and are responsible for the altered sensitivity. It is, however, possible to alter the composition of the membrane phospholipids of cultured mammalian cells by dietary means (Gerner et al., 1962; Spector et al., 1979; Konings, 1985). Clearly if the plasma membrane is an important site of hyperthermic damage in mammalian cells then any changes produced in its membrane lipid composition might well be expected to influence events that occur under heat stress.

Early studies of the lipid nutrition and metabolism of cultured cells indicated that most cultured cells were capable of synthesising essentially all of the lipids they require from water soluble compounds present in the culture medium. However, since the culture medium typically contained serum which is rich in lipids, including lipoproteins and free fatty acids, de novo synthesis of lipids was found to be inhibited by exogenous lipids taken up from the culture medium. Initially it was thought that because phospholipids exist within membranes, their fatty acid composition would be closely regulated and unlikely to reflect the composition of the lipids contained in the culture medium. However, Geyer et al. (1962) working with mouse L fibroblasts soon showed that this was not the case. These workers demonstrated that in the presence of chemically defined medium, L fibroblast phospholipids were devoid of polyunsaturated fatty acids such as 18 : 2. However, when these cells were exposed to dialysed serum in the culture medium for 144 hours the phospholipids were found to contain approximately 15% of 18 : 2 which replaced 18 : 1. Since 1962, the recognition that fairly extensive membrane lipid modifications could be produced by varying the composition of the culture fluid has led to a great deal of work which has utilised this approach

to study the role of lipid composition in membrane function and the response of cells to hyperthermic treatment.

The use of cultured cells offers the capability of modifying fatty acid composition of membrane lipids more rapidly than can be achieved *in vivo*, that is within a few hours rather than days. Also, since the cells derive from the same population, the individual variation experienced in dietary studies can be avoided. In addition, changes in fatty acid composition that can be brought about in cell culture may often be of much greater magnitude than can be achieved by dietary means, as the processing of ingested fatty acids that occurs during the synthesis of plasma lipoproteins reduces the extremes of variation that can be achieved *in vivo*.

Mammalian cells in culture appear to have a virtually unlimited capacity to incorporate and accumulate exogenous unsaturated fatty acids (Rosenthal, 1987). There is no apparent regulatory mechanism to limit fatty acid uptake, instead excess fatty acyl groups are stored as triacylglycerols (Rosenthal, 1980). Accumulation of cytoplasmic lipid droplets can reach massive proportions causing cells to round up and even rupture (Geyer, 1967). However, minute fat droplets are often present in the extranuclear space of cultured cells even in the absence of an exogenous fatty acid supply, and most cells will tolerate a moderate increase in triacylglycerol reserves without any apparent deleterious effects. Studies have shown (Schneeberger et al., 1971) that the excess neutral lipid droplets readily disappear when cells are subsequently cultured in medium without exogenous fatty acid.

Saturated fatty acids, on the other hand, often cause toxicity effects at concentrations within the medium which are non-toxic for unsaturated fatty acids (Urade and Kito, 1982). This toxicity has been attributed to accumulation of crystalline solid neutral lipids (Goto et al., 1986) or to increased saturation of membrane phospholipids with a resultant damaging effect on membrane physical state (Doi et al., 1978).

Three major problems were anticipated during early attempts to achieve net replacements of fatty acids in membranes. Firstly, it was thought that cells would possess adequate enzymic mechanisms to control fatty acyl composition

so that intervention would be needed to prevent this. Secondly, it was felt that since most cultured cells required serum, which contains a variety of lipids, this would limit the extent to which lipid composition of the incubation medium could be varied. Thirdly, it was thought that the toxic nature of free fatty acids that had been observed by a number of workers (Geyer, 1967; Moskowitz, 1967) might cause unacceptable levels of damage to cells. As a result the early studies with lipid supplements were performed with mouse LM cells, a variant of the L fibroblast that could be grown in a serum-free medium. Fatty acid biosynthesis was inhibited by the presence of a biotin analogue, whilst the likelihood of fatty acid toxicity was reduced by using various fatty acid esters of Tween. In this way large differences in the fatty acid composition of the LM cell phospholipids were produced (Williams et al., 1974). In other experiments , again with mouse fibroblast LM cells, the linoleic acid content of phospholipid fatty acyl groups was raised from undetectable levels to 37% by supplementing a serum-free medium with linoleic acid bound to albumin (Glaser et al., 1974). Fatty acyl modifications have also been produced in 3T3 cells by growing them in a culture medium containing lipid depleted serum, supplemented with specific fatty acids (Horwitz et al., 1974).

However, it has since become apparent that although these methods can be useful in certain cases, many diploid cells cannot be grown in adequate amounts in either a serum-free or lipid-depleted medium and in any case a wide variety of cells will readily incorporate net amounts of exogenous fatty acid into their membrane phospholipids in the presence of serum. For example, extensive modifications in human skin fibroblast phospholipid fatty acyl chain composition have been produced by adding specific fatty acids to the usual growth medium containing 10% (v/v) foetal bovine serum (Spector et al., 1979). In addition, it has been found that overt toxicity can be avoided fairly easily by using low amounts of fatty acids.

The extent of fatty acyl chain modifications of membrane lipids is known to depend on the time of exposure to the supplemental fatty acid and its concentration. For example, when Y79 retinoblastoma cells were supplemented with $30\mu M$ docosahexaenoic acid (22 : 6) for various times up to 72 hours, the largest phospholipid fatty acyl compositional changes occurred during the first 48 hours.

Furthermore, in 72 hour exposures to a range of 22 : 6 concentrations, maximum enrichment with 22 : 6 in membrane lipids was produced with supplemental concentrations of less than $40\mu M$ (Spector and Yorek, 1985).

Reports by a number of workers have suggested a correlation between thermosensitivity and membrane fatty acid composition. Much of the early work concentrated on the use of bacterial cells. Using the *Escherichia coli* mutant K1060, which is defective both in its ability to synthesise and to degrade unsaturated fatty acids, Yatvin (1977) demonstrated that cells enriched with the polyunsaturated fatty acid linolenic acid (18 : 3) were more susceptible to thermal killing than were cells enriched with the monounsaturated acid, oleic acid (18 : 1). In addition, Overath et al. (1970) found that the 18 : 3 supplemented *E.Coli* K1060 cells were incapable of growth at temperatures greater than $40°C$, whereas the 18 : 1 supplemented cells could grow at temperatures up to $45°C$.

Similar observations have been made with mammalian cells. Hidvegi et al. (1980) working with murine P388 cells showed that when such cells were grown in animals fed a diet rich in polyunsaturated fatty acids, they were more thermosensitive than cells grown in animals fed a diet rich in saturated fatty acids. Konings (1985) modified mouse fibroblast LM cells with respect to the content of their polyunsaturated fatty acyl chains of the phospholipids and showed not only that increased levels of polyunsaturated fatty acids in the plasma membrane led to enhanced thermosensitivity, but that they also led to an increase in the fluidity of the membranes; a finding that has been reported by other workers (King et al., 1977; Yatvin, 1977; Guffy et al., 1982) and has led to the hypothesis that the fluidity of membranes might be a major factor contributing to the death of cells exposed to hyperthermia. This hypothesis is, however, disputed by a number of workers, in particular Lepock and collaborators (Lepock et al., 1981; Massicotte-Nolan, 1981; Lepock, 1982, Lepock et al., 1983, 1988, 1989) who suggest that membrane protein denaturation rather than membrane lipid fluidity is the key factor leading to hyperthermic cell death as shall be discussed in Chapter 4.

This chapter has a number of aims. Firstly, to characterise the hyperthermic response of HTC cells over a range of temperatures using the two assay procedures for cell survival described in Chapter 2. Characterisation of the hyperthermic

response will involve a consideration of factors such as the presence of polyene antibiotics (Hahn et al., 1977), serum (Van Dongen and Van Wijk, 1986), pH (Nielsen and Overgaard, 1979) and cell passage number which have been reported to influence the hyperthermic response of cells. Secondly, surface morphology studies of heated cells will be considered in an attempt to correlate membrane surface modifications with hyperthermic treatment. Thirdly, it aims to establish a suitable procedure for supplying fatty acids to HTC cells. Finally, it aims to establish whether there is any evidence for a correlation between fatty acid supplementation of HTC cells and their thermosensitivity.

3.2 Materials and Methods

3.2.1 Hyperthermic Studies

(a) Flasks: Clonogenic assay

Cells (3×10^5) were seeded into $10ml$ of growth medium in $25cm^2$ flasks on day 0 and incubated at $37°C$ under a humid atmosphere of air/CO_2 (19 : 1, v/v). In the absence of supplementation, the medium was changed in the flasks the following day (day 1) and on day 3. In supplementation studies cells were exposed to various concentrations of FFA supplemented medium on either day 1, day 2 or day 3 depending on the period of supplementation required. In both cases exponentially growing cells were harvested on day 4 by exposure to EDTA solution as described in Chapter 2, Materials and Methods, section 2.2.2(a). A known number of cells (typically 200/flask) were then seeded into $10ml$ of medium lacking fungizone (Hahn et al., 1977) 'heating medium' in $25cm^2$ flasks and the cells were allowed to attach to the flask surface at $37°C$ for three hours.

The flasks were then sealed and thermoequilibrated in a water bath at $37°C$ before rapid transfer to a water bath at the hyperthermic temperature ($\pm 0.1°C$). Equilibration to the higher temperature was complete within 5 minutes and this lag period was included in the total heating time. After heating, the unsealed flasks were returned to the $37°C$ incubator. The medium was replaced 1, 5 and 8 days later and on day 9 the cells were fixed and stained in trypan blue and

the colonies counted as described in Chapter 2, Materials and Methods, section 2.2.3(d).

(b) Plates: Colorimetric assay

Cells (5×10^3/well) were seeded into 24-well plates in $1ml$ of growth medium on day 0 and incubated at $37°C$ under a humid atmosphere of air/CO_2 (19 : 1, v/v). In the absence of supplementation, the medium was changed on day 1. In supplementation studies, cells were exposed to various concentrations of linoleic acid-supplemented medium on day 1, at a time calculated to ensure a 36 hour supplementation period prior to heat treatment. In both cases, the medium was replaced on day 3 with medium containing $10mM$ Hepes pH 7.4, but lacking fungizone. The plates, containing exponentially growing cells were returned to the incubator to gas for 20 minutes. After gassing the plates were sealed and thermoequilibrated at $37°C$ before rapid transfer to a water bath set $1.5°C$ higher than the required temperature for 5 minutes before final transfer to a water bath at the desired hyperthermic temperature ($\pm 0.1°C$). This protocol reduced the lag time to approximately that determined for flasks. After heating, unsealed plates were returned to $37°C$. The medium was changed the following day (day 4) and cell survival was assessed on day 6 by the colorimetric assay described in Chapter 2, section 2.2.4(b).

3.2.2 Surface Morphology Studies of Heated Cells

Cells (3×10^5) were seeded into $25cm^2$ flasks containing glass coverslips on day 0 in $10ml$ growth medium, and incubated at $37°C$ under a humid atmosphere of air/CO_2 (19 : 1, v/v). The medium was changed on day 1. On day 3, after replacing the medium with heating medium and gassing for 20 minutes in the $37°C$ incubator, the flasks were heated as described in section 3.2.1(a), for various periods of time up to 80 minutes at $43.5°C$.

Following treatment, the cells on coverslips in each flask were washed once with $10ml$ calcium and magnesium-free phosphate buffered saline (Ca^{2+}-, Mg^{2+}-free PBS). Fixation techniques were based on the methods of Karnovsky (1965). Details of the fixative are given in Chapter 2, Materials and Methods, section 2.2.2(b). The Ca^{2+}-, Mg^{2+}-free PBS used to wash the cells was replaced with

$10ml$ of Karnovsky fixative, containing glutaraldehyde 2.1% (w/v) in sodium cacodylate $(0.08M)$, and left for 1 hour at $4°C$. The cells were then post-fixed on the coverslips in the $25cm^2$ flasks in $10ml$ osmium tetroxide (1%, w/v) in sodium cacodylate $(0.1M)$, dehydrated through the CO_2 critical point and sputter coated with gold palladium by the methodology described in Chapter 2, Materials and Methods, section 2.2.2(b). The samples were then examined in a Stereoscan 800 scanning electron microscope.

3.2.3 Fatty Acid Supplements

Culture medium was supplemented with either linoleic acid (18 : 2) or stearic acid (18 : 0) which were obtained as their sodium salts in a > 99% pure form and were added to the newborn bovine serum used to prepare the growth medium. Stock solutions of free fatty acid (approximately $2mM$ final concentration) in newborn bovine serum (NBS) were prepared. The free fatty acid sodium salt was dissolved in a small volume of distilled water ($3ml/50ml$ of supplement prepared). Dissolution of the salt was performed at $40°C$ for linoleic acid and at $80°C$ for stearic acid. Once the salts had dissolved, an appropriate volume of NBS warmed to $40°C$ was added with mixing, at $40°C$. The resulting NBS-free fatty acid solution was sterilised through a $0.22\mu m$ filter and then aliquots ($1ml$), in glass vials, were gassed with nitrogen, sealed and stored at $-20°C$ until required.

Supplemented medium was prepared by substituting normal NBS in the growth medium with an appropriate volume of NBS that had been supplemented with $2mM$ free fatty acid (NBS-FFA). The water content of the NBS-FFA was compensated for when preparing growth medium by adding additional NBS.

3.2.4 Free Fatty Acid Assay

Free fatty acid assays of normal and supplemented NBS were carried out by the method of Antonis (1965) with some modifications. The method is based on the production of the copper salt of the fatty acids in chloroform followed by an estimation of the copper content of the organic phase by reaction with zinc dibenzyldithiocarbamate ($ZnDDC$). Silicic acid ($1.2 \pm 0.1g$), that had been activated at $110°C$ for 1 hour prior to use, was slurried with $7.5ml$ isopropyl ether

in capped vials. The isopropyl ether was passed through a column of activated alumina prior to use to remove peroxides. Serum ($0.3ml$) or serum-FFA ($0.3ml$) was added to the vials and the mixture was mixed vigorously for 1.5 minutes. The silicic acid acted as a phospholipid adsorbant, removing phospholipids which were a potential source of interference, without affecting the levels of free fatty acids. Once the contents of the vials had settled, a known volume ($4ml$) of the isopropyl ether supernatant was removed and transferred to glass centrifuge tubes which had been previously 'siliconised' using dimethyldichlorosilane (0.5%, v/v) in heptane. The isopropyl ether was evaporated to dryness under nitrogen in a $40°C$ water bath and the residue in each tube was redissolved in $5ml$ chloroform. 'Copper reagent' ($2.5ml$) consisting of 3.8% (w/v) copper sulphate, pentahydrate, $0.45M$ triethanolamine and $0.05M$ acetic acid in final concentrations was added immediately and the tubes mixed thoroughly for 45 seconds. The tubes were then spun at low speed for 10 minutes. After spinning, the excess upper aqueous phase was carefully removed by aspiration with a pasteur pipette. A $3ml$ aliquot of the chloroform extract was placed into a clean test tube and the colour was developed by the addition of $0.5ml$ of $ZnDDC$ (0.3%, w/v) in chloroform. The tubes were mixed and the absorbance read at $440nm$ after 15 minutes at room temperature.

Standard curves over the range 0-0.6μmoles palmitate were produced using $12mM$ palmitic acid in chloroform and PBS to act as aqueous phase. Assays were performed in duplicate; blank assays and standard assays being included in each series of analyses.

The 'copper reagent' and $ZnDDC$ reagent were stored in the dark at $4°C$ and replaced every 2 months.

3.2.5 Fatty Acid Toxicity Studies

(a) Flasks

Cells ($3 \times 10^5/25cm^2$ flask) were seeded into $10ml$ of growth medium containing either 5% (v/v) or 10% (v/v) serum on day 0 and incubated at $37°C$ under a humid atmosphere of air/CO_2 (19 : 1, v/v). The medium was changed the following day. To produce modifications in membrane fatty acid composition,

cells were exposed on day 2 to growth medium containing either 5% (v/v) or 10% (v/v) serum, supplemented with varying concentrations of linoleic acid or stearic acid, for varying time periods up to 48 hours.

The effect of fatty acid supplementation in flasks was monitored in terms of cell growth which was assessed by cell counts obtained with the Coulter Counter as described in Chapter 2.

(b) Plates

Cells (5×10^3/well) were seeded into 24-well plates in $1ml$ of growth medium on day 0 and incubated at $37°C$ under a humid atmosphere of air/CO_2 (19 : 1, v/v). The cells were exposed to linoleic acid supplemented growth medium the following day for varying periods up to 72 hours. In both plate and flask supplementation procedures the supplemented medium was replaced every 24 hours to prevent depletion of the fatty acid supplement.

The effect of fatty acid supplementation in plates was monitored in terms of cell number, which was assessed by the colorimetric assay described in Chapter 2, Materials and Methods, section 2.2.4(b).

3.2.6 Cytoplasmic Lipid Droplet Studies

Cells (5×10^3/well) were seeded into 24-well plates containing glass coverslips in $1ml$ of growth medium on day 0 and incubated at $37°C$ under a humid atmosphere of air/CO_2 (19 : 1, v/v). The medium was changed the following day, and on the second day after seeding the cells were exposed to $80\mu M$ linoleic acid supplemented growth medium. Accumulation of cytoplasmic lipid droplets, produced by the uptake of the fatty acid by cells on coverslips was monitored microscopically. The study employed an inverted Nikon Diaphot microscope that was fitted with differential interference contrast Normarski optics. Coverslips were placed on the microscope stage in perspex holders containing growth medium previously warmed to $37°C$ with or without supplement and photographed with a Nikon camera.

Disappearance of the excess neutral lipid droplets was also monitored microscopically after returning cells to normal growth medium following periods of

supplementation.

3.3 Results

Serum is rich in lipids including lipoproteins and free fatty acids and is highly variable in its composition with respect to hormones and other undefined substances (Olmsted, 1967; Honn et al., 1975). In consequence, preliminary investigations were undertaken to determine the conditions that allowed the removal of, or a reduction in the need for serum for cell survival and growth in culture, by the use of defined culture medium (DCM) and delipidated serum. The purpose of such investigations was to produce a culture medium that could be supplemented with particular fatty acids to produce alterations in the fatty acid profile of membrane phospholipids of the cells.

Attempts to adopt techniques reported by other workers for the growth of cells in serum-free medium presented a number of problems. For example, the 'improved minimal essential medium zinc option' type of DCM (Richter, 1970; Thompson et al., 1975) yielded zero plating efficiency and so was not further used. The use of delipidated serum proteins (Rothblat et al., 1976), which avoids introducing the wide spectrum of free fatty acids and other lipids normally present in most types of serum, was not successful since these delipidated serum proteins were highly insoluble in the culture medium at the recommended level of $8mg/ml$. Lyophilised serum proteins (Wood, 1973) were equally insoluble in the culture medium. Lipid free serum (Cham and Knowles, 1976) produced by extracting serum with a diisopropyl ether : butanol mixture (60 : 40, v/v) was more successful in terms of its solubility in culture medium. However, as shown in figure 3.1, since HTC cells failed to grow successfully in the absence of unprocessed serum, the use of delipidated serum proteins could not be pursued.

Since serum had to be maintained in the culture medium for the successful growth of HTC cells an attempt was made to reduce the level of serum normally present in the culture medium from 10% (v/v) to 5% (v/v), so that exposure of HTC cells to the 'unknown' fatty acid content characteristic of serum would be reduced in subsequent supplementation experiments.

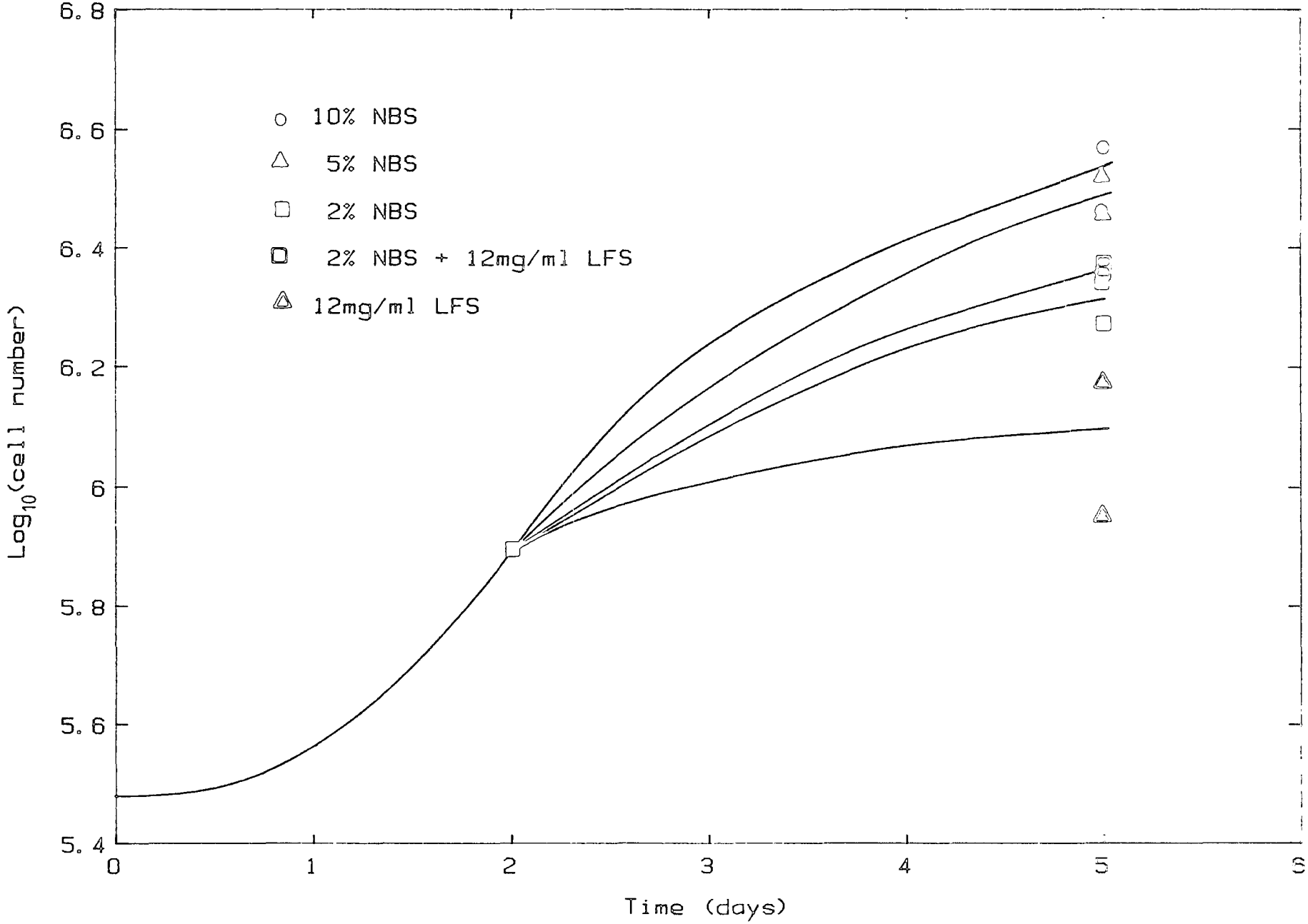
Initially growth of HTC cells did not appear to be markedly affected when

Figure 3.1

The effect of medium composition on cell growth

Flasks were seeded with 3×10^5 cells in $10ml$ of growth medium on day 0 and maintained in an incubator at $37°C$ under a humid atmosphere of air/CO_2 (19 : 1, v/v). The medium was changed on day 1. On day 2 cells were exposed to growth medium containing different levels of newborn bovine serum (NBS) and/or lipid-free serum (LFS) as indicated on the graph. The medium was changed on day 3 and cell growth was assessed on day 5 by the Coulter Counter method described in Chapter 2, Materials and Methods, section 2.2.3(c).

The points represent individual results derived from 2 flasks in a single experiment.


10% NBS
5% NBS
2% NBS
2% NBS + 12mg/ml LFS
12mg/ml LFS
Log10(cell number)
6.8
6.6
6.4
6.2
6
5.8
5.6
5.4
0
1
2
3
4
5
Time (days)

the serum level in the culture medium was reduced from 10% (v/v) to 5% (v/v). Consequently, supplementation procedures were conducted in growth medium containing 5% (v/v) newborn bovine serum (NBS) in the early stages of the study. Unfortunately however, after a few months of culture, cell growth began to deteriorate at 5% (v/v) NBS levels and serum levels had to be returned to 10% (v/v) in the growth medium. As a result, all thermal sensitivity studies of normal and supplemented HTC cells were conducted in growth medium containing 10% (v/v) NBS.

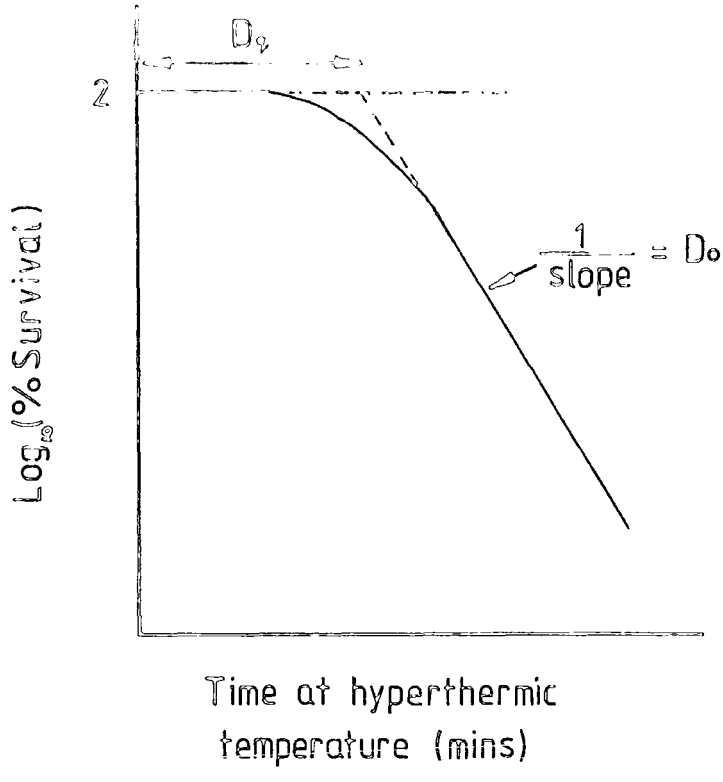
The vast majority of hyperthermic studies *in vitro* have been based on clonogenic assays which tend to produce dose response curves of the logarithm of the surviving cell fraction versus time at hyperthermic temperature that are similar to those obtained after exposure to ionising radiation. Such curves are characterised by an initial shoulder followed by a phase of logarithmic cell killing, and as a consequence, results are often considered in terms of the two parameters D_0 and D_q (see figure 3.2). In hyperthermic inactivation studies D_0 describes the linear potion of the curve and is the reciprocal of the slope, i.e. the duration in minutes at a particular temperature needed to reduce the survival to $1/e$ (37%) of the initial value (Bhuyan, 1979). D_q, which is often referred to as the 'quasithreshold dose' is used to describe the size of the shoulder and may be defined as the time in minutes from the start of therapy obtained by back extrapolating the linear part of the curve to the Log_{10} %survival = 2 axis (Harris et al., 1977).

Before the hyperthermic response of HTC cells in terms of the clonogenic assay and colorimetric assay were characterised in detail, studies were undertaken to ensure that factors other than temperature such as pH, cell passage number and the presence of particular medium components etc. were not influencing the survival curves obtained.

Table 3.1 shows the variation in pH of heating medium with and without $10mM$ Hepes pH 7.4, that occurred over a $3\frac{1}{2}$ hour period at $43°C$ in the absence of cells. It can be seen that whilst the pH values at $43°C$ do not alter significantly from the incubator control values in the case of the flasks, there is a difference in the case of the 24-well plates. This difference may arise because flasks contained a larger volume of medium $(10ml)$ than the plates $(1ml/\text{well})$.

Figure 3.2

Parameters describing hyperthermic survival curves derived from clonogenic assays



D_0:

Reciprocal of the slope of the linear portion of the survival curve. The duration in minutes at a particular temperature needed to reduce the survival to $1/e$ (37%) of the initial value.

D_q:

Describes the size of the shoulder and is the time in minutes from the start of therapy obtained by back extrapolating the linear portion of the survival curve to the Log_{10} %survival = 2 axis.

Table 3.1

The influence of temperature on pH values recorded in 24-well plates and 25cm^2 flasks

Heating medium was prepared in the absence or presence of 10mM Hepes, pH 7.4. Heating medium without Hepes was added to six 25cm^2 flasks (10ml /flask). Heating medium with and without Hepes was added to the wells of six 24-well plates. After gassing for 60 minutes in the 37°C incubator under a humid atmosphere of air/CO_2 (19 : 1, v/v), one flask and plate were maintained in the incubator, one flask and plate were maintained at 37°C in a water bath and the remaining plates and flasks were heated at 43°C in a water bath. pH determinations were made at the time points stated using a Phillip Harris digital pH meter. The results represent data from a single experiment.

Temperature (°C)	Time (mins)	pH value		
		Flask minus Hepes	Plate minus Hepes	Plate plus Hepes
37 Incubator	210	7.41	7.40	7.43
37 Bath	210	7.42	7.65	7.60
43 Bath	30	7.48	7.95	7.71
43 Bath	90	7.55	7.84	7.67
43 Bath	150	7.47	7.84	7.61
43 Bath	210	7.44	7.89	7.57

The addition of $10mM$ Hepes buffer, pH 7.4, reduced the extent of the pH change that occurred and was consequently always added to heating medium used in plate hyperthermic studies.

Figure 3.3 shows the effect of heating cells in flasks over a range of temperatures in the absence or presence of fungizone ($2.5\mu g/ml$) in the growth medium. Regression lines fitted to the linear portions of the survival curves suggest that the presence of fungizone did increase the thermal sensitivity of the cells. This agrees with Hahn et al. (1977), especially at higher temperatures. For example, at $45°C$ the value of D_0 is decreased by approximately 10 minutes by the presence of fungizone. As a result, fungizone was excluded from growth medium that was used in subsequent flask and plate heating experiments.

Figure 3.4 shows the results of a single experiment run to monitor the effect of serum concentration on the hyperthermic response of cells heated in flasks at $43.5°C$. Regression lines fitted to the linear portions of the survival curves (with regression correlation coefficients, $|r| > 0.83$) suggest that the serum level does affect the thermal sensitivity of cells with lower serum levels leading to an increased sensitisation to heat. In all subsequent experiments that were conducted cells were routinely grown and heated in growth medium containing 10% (v/v) NBS.

Figure 3.5 shows the effect of passage number on the hyperthermic cell survival levels recorded at $42°C$ and $43°C$ from duplicate flasks run in single experiments. It is evident that a temperature of $42°C$ does not markedly affect cell survival. In addition, both at $42°C$, and also at $43°C$ where cell survival is reduced by approximately 70% after 90 minutes, it can be seen that passage number does not appear to have a marked effect on cell survival whilst cells that had been subcultured a total of 19 times (passage 19 cells) appear to be slightly less sensitive to heat than cells that had been subcultured a total of 9 times (passage 9 cells). Statistical analysis reveals that this difference is not significant. However, since only two measurements were made at each time point, to avoid the possibility of variation in results being due to passage number, subsequent hyperthermic studies were restricted to cells that had been subcultured fewer than 10 times in the laboratory.

Figure 3.3

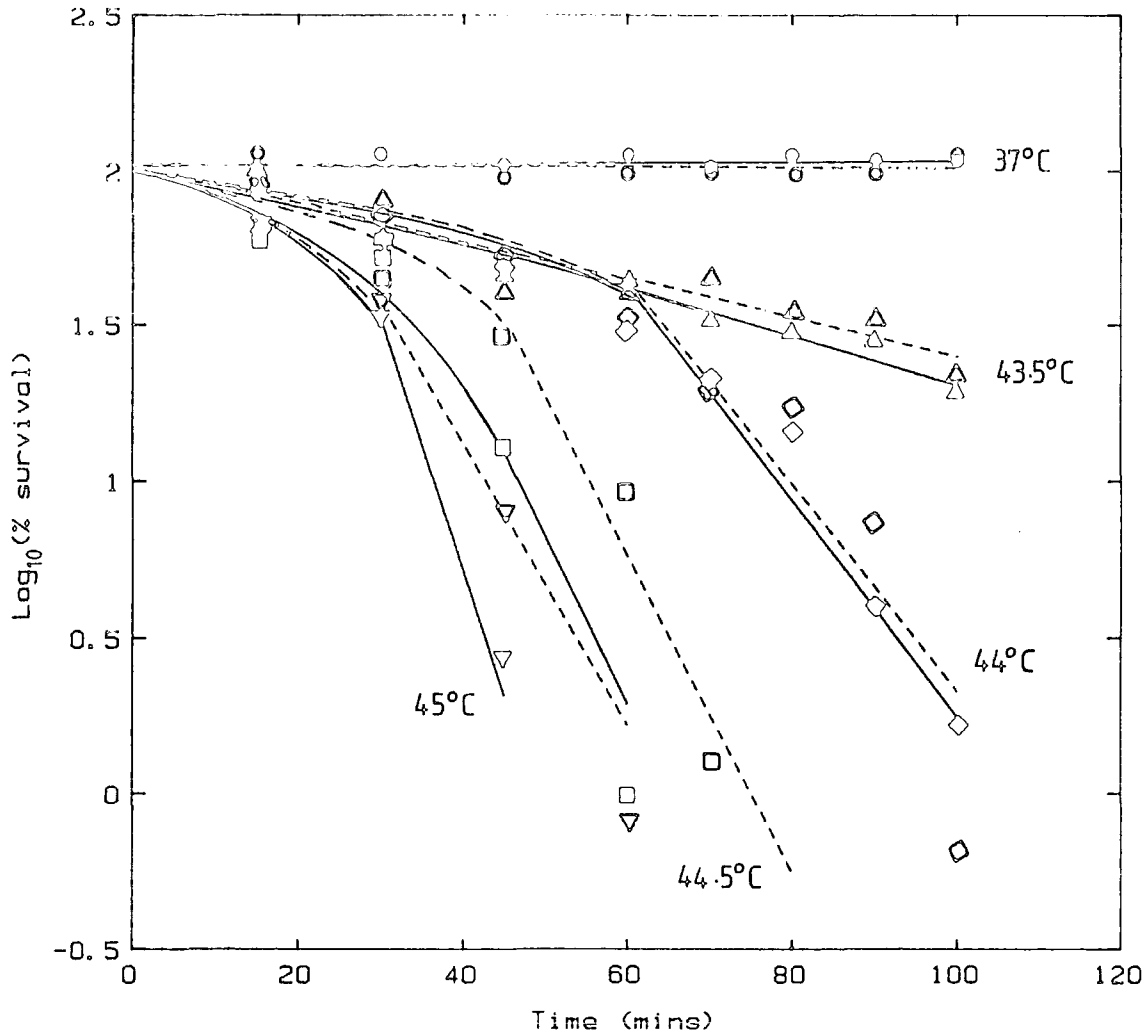
The effect of fungizone on the thermal sensitivity of HTC cells

Exponentially growing HTC cells were harvested using EDTA solution and 200 cells/25cm^2 flask were seeded into 10ml of growth medium in the presence or absence of fungizone (2.5$\mu g/ml$). Flasks were incubated at 37°C under a humid atmosphere of air/CO_2 (19 : 1, v/v) for three hours and then sealed and heated at various temperatures (37°C to 45°C) for various periods of time up to 100 minutes. Following heating, unsealed flasks were returned to the 37°C incubator. The medium was replaced 1, 5 and 8 days later with normal growth medium and cell survival was assessed on day 9 by the clonogenic assay described in Chapter 2, section 2.2.3(d).

The data points represent mean values derived from two flasks in a single experiment run over the temperature range. Best fit regression lines have been fitted to the linear portions of the survival curves using individual flask results and the straight line equation $y = a + bx$.

Correlation coefficients of the best fit regression lines and values of D_0 are given below.

Temperature (°C)	D_0 (mins)		Correlation coefficient	
	without fungizone	with fungizone	without fungizone	with fungizone
43.5	155.0	128.2	−0.780	−0.883
44	30.0	29.4	−0.875	−0.929
44.5	19.7	18.9	−0.976	−0.999
45	22.2	12.5	−0.998	−0.885



Temperature ($°C$)	without fungizone	with fungizone
37	----- ○	—— ○
43.5	----- △	—— △
44	----- ◇	—— ◇
44.5	----- □	—— □
45	----- ▽	—— ▽

Figure 3.4

The effect of serum on the thermal sensitivity of HTC cells

Exponentially growing HTC cells cultured in growth medium containing either 5% (v/v) or 10% (v/v) newborn bovine serum (NBS) were harvested using EDTA solution and 200 cells/$25cm^2$ flask were seeded into $10ml$ of heating medium containing either 5% (v/v) or 10% (v/v) NBS as indicated on the graph. Flasks were incubated at $37°C$ under a humid atmosphere of air/CO_2 (19 : 1, v/v) for three hours and then sealed and heated at $43.5°C$ for various periods of time up to 100 minutes. Following heating, unsealed flasks were returned to the $37°C$ incubator. The medium was replaced 1, 5 and 8 days later with normal growth medium and cell survival was assessed on day 9 by the clonogenic assay described in Chapter 2, section 2.2.3(d).

The data is derived from a single experiment. Best fit regression lines have been fitted to the linear portions of the survival curves using the straight line equation $y = a + bx$.

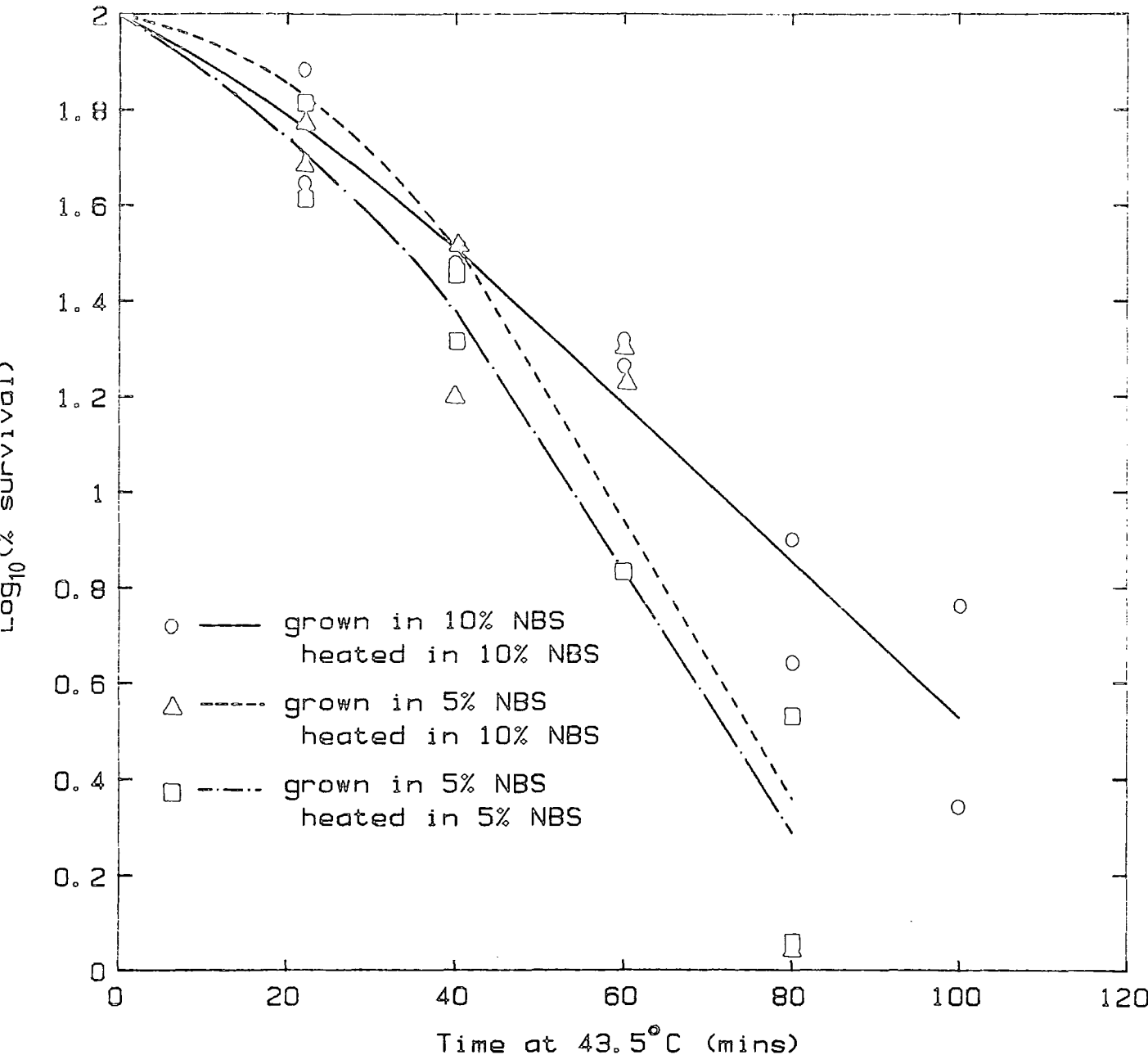
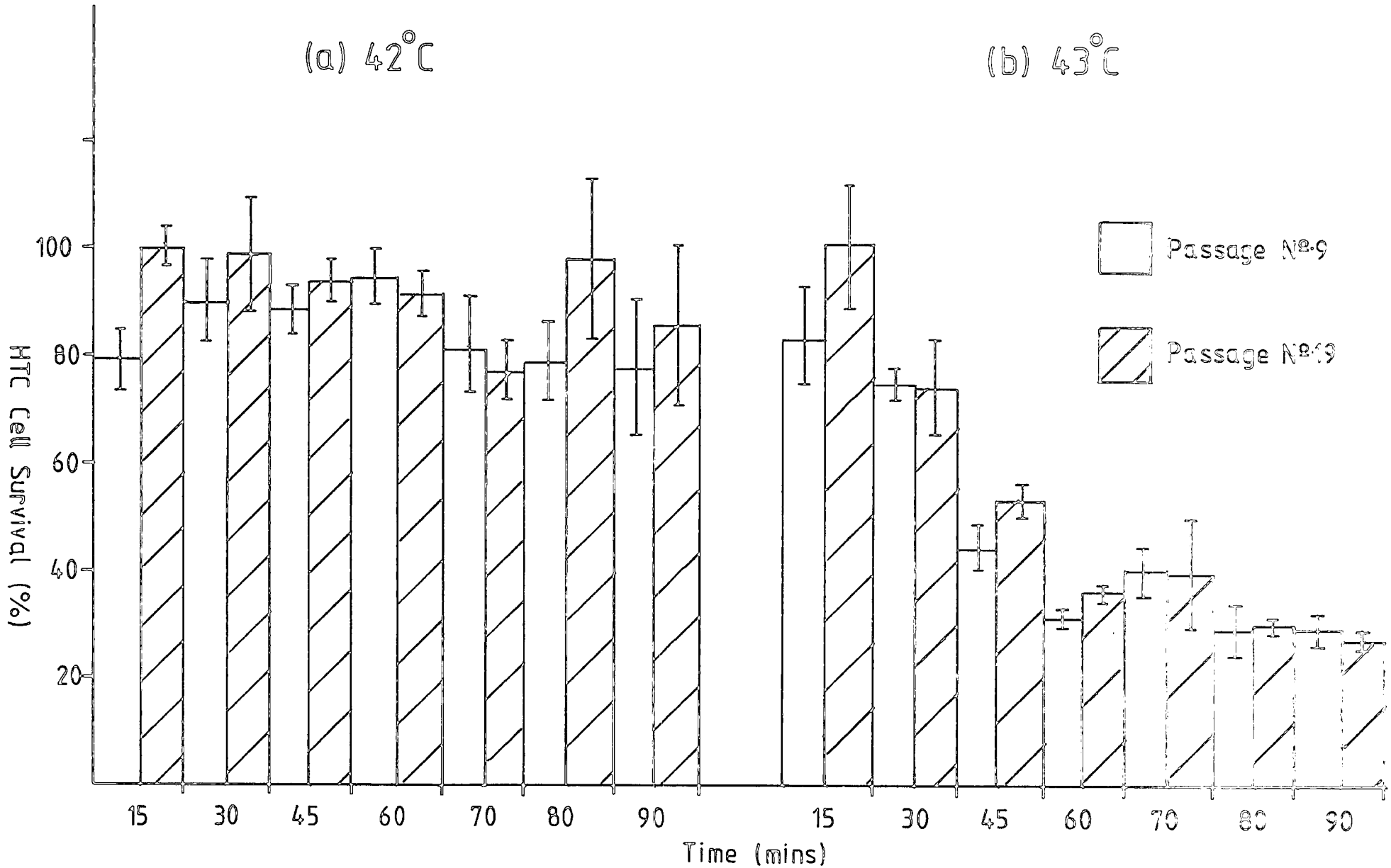

2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Log10(% survival)
0
20
40
60
80
100
120
Time at 43.5°C (mins)
grown in 10% NBS
heated in 10% NBS
grown in 5% NBS
heated in 10% NBS
grown in 5% NBS
heated in 5% NBS

Figure 3.5

The effect of passage number on the thermal sensitivity of HTC cells

Exponentially growing HTC cells (passage 8 and passage 18) were harvested using EDTA solution and 200 cells/$25cm^2$ flask were seeded into $10ml$ of heating medium and incubated at $37°C$ under a humid atmosphere of air/CO_2 (19 : 1, v/v) for three hours. After sealing, flasks were heated at $42°C$ or $43°C$ for various periods of time up to 100 minutes. Following heating, unsealed flasks were returned to the $37°C$ incubator. The medium was replaced 1, 5 and 8 days later with growth medium and cell survival was assessed on day 9 by the clonogenic assay described in Chapter 2, section 2.2.3(d).

The bar chart shows the mean values derived from two flasks at each time point in a single experiment at each temperature.


(a) 42°C
(b) 43°C
Passage No.9
Passage No.19
HTC Cell Survival (%)
100
80
60
40
20
15
30
45
60
70
80
90
15
30
45
60
70
80
90
Time (mins)

Having 'optimised' hyperthermic conditions as far as possible, a morphological study of HTC cells heated in flasks on glass coverslips at $43.5°C$ for various times up to 80 minutes was conducted. The results of the study are shown in figure 3.6. Figures 3.6(a) to (f) show low power surface views of monolayer HTC cells during the heating process. Under normal culture conditions (figure 3.6(a)) it can be seen that the cells spread out over the substrate surface as a fairly flat, even layer with little evidence of cellular boundaries between adjacent cells. After 15 minutes at $43.5°C$ (figure 3.6(b)) it is evident that cells are beginning to separate from each other. Over the next 55 minutes (figures 3.6(c), (d) and (e)) this separation continues and cells start to round up so that by 70 minutes (figure 3.6(e)) the originally continuous sheet of cells consists of well separated rounded cells. By 80 minutes (figure 3.6(f)) cells are also beginning to show signs of detaching from the substrate surface.

Figures 3.6(g) to (l) show high power surface views of cells under the same conditions. Under normal culture conditions (figure 3.6(g)) the cell surface is covered with numerous microvilli and cells exist in close association with surrounding cells. After 15 minutes (figure 3.6(h)) whilst cell surfaces still bear microvilli, the cell boundaries are becoming evident as cells start retracting cellular processes as they round up. By 30 minutes (figure 3.6(i)) there is obvious cell separation, whilst at 60 minutes (figure 3.6(j)) the rounded nature of many of the cells is apparent. At this stage cell surfaces are still characterised by the presence of microvilli. However, as heating continues these microvilli are lost (figure 3.6(k)) and by 80 minutes (figure 3.6(l)) the cell surface is also characterised by the presence of protrusions from the cell surface commonly described in the literature as 'blebs'.

Figures 3.7 and 3.8 show the hyperthermic survival curves generated for HTC cells over a range of temperatures from $42.5°C$ to $45°C$ based on results obtained with the clonogenic assay and colorimetric assay respectively. In the clonogenic assay, cells were heated in flasks and cell survival was assessed in terms of the cells' reproductive ability at 9 days after heating. In the colorimetric assay on the other hand, cells were heated in plates and cell survival was measured at 3 days after heating and cell survival was assessed in terms of the cells' ability to cleave MTT (3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide)

Figure 3.6

Surface morphological changes associated with hyperthermic treatment of HTC cells

Flasks containing glass coverslips were seeded with 3×10^5 cells in $10ml$ of medium on day 0 and maintained in an incubator at $37°C$ under a humid atmosphere of air/CO_2 (19 : 1, v/v). The medium was changed on day 1. On day 3 the cells on coverslips were heated at $43.5°C$ for various periods of time up to 80 minutes and processed as described under Materials and Methods, section 3.2.2, for examination in a Stereoscan 800 scanning electron microscope.

Low power surface views

(a) Control HTC cells

(b) 15 minutes at $43.5°C$

(c) 30 minutes at $43.5°C$

(d) 60 minutes at $43.5°C$

(e) 70 minutes at $43.5°C$

(f) 80 minutes at $43.5°C$

The bar in each micrograph represents $20\mu m$.

High power surface views

(g) Control HTC cells

(h) 15 minutes at $43.5°C$

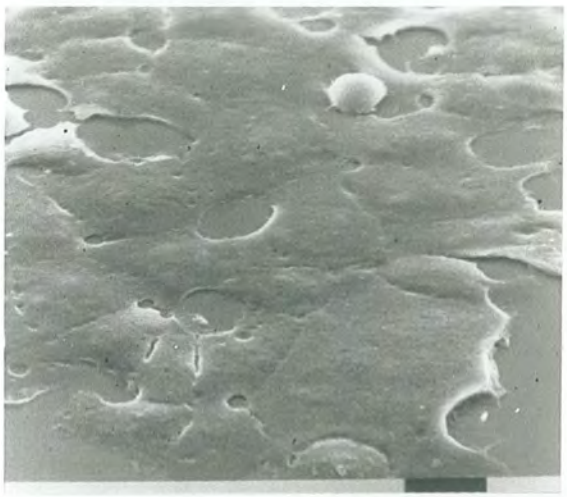
(i) 30 minutes at $43.5°C$

(j) 60 minutes at $43.5°C$

(k) 70 minutes at $43.5°C$

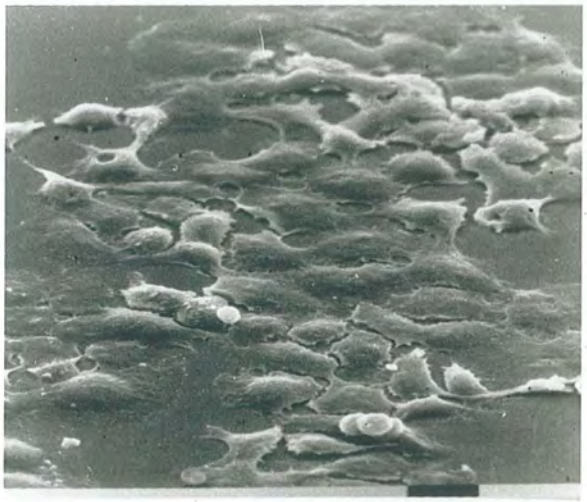
(l) 80 minutes at $43.5°C$

The bar in each micrograph represents $4\mu m$.



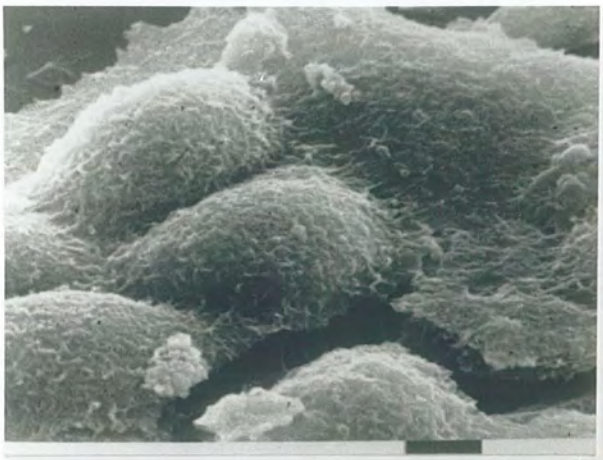
a

b

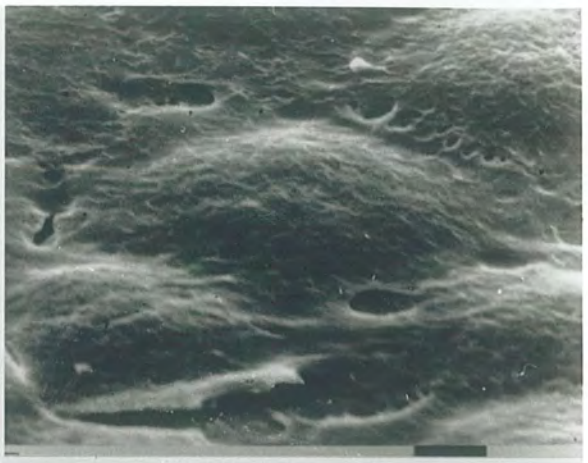


c

d



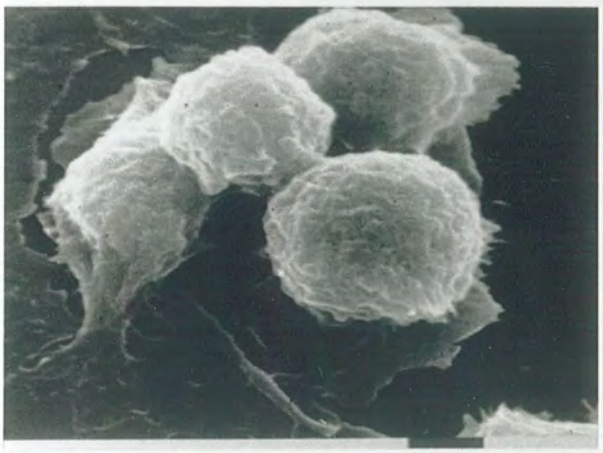
g



h

i

j



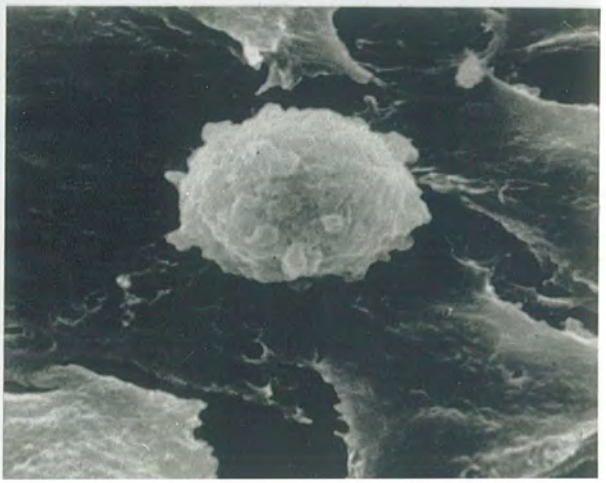


Figure 3.7

Hyperthermic cell death of HTC cells heated in flasks, assessed by the clonogenic assay

Exponentially growing HTC cells were harvested using EDTA solution and 200 cells/$25cm^2$ flask were seeded into $10ml$ of heating medium and incubated at $37°C$ under a humid atmosphere of air/CO_2 (19 : 1, v/v) for three hours. After sealing, flasks were heated at various temperatures ($37°C$ to $45°C$) for various periods of time up to 100 minutes. Following heating, unsealed flasks were returned to the $37°C$ incubator. The medium was replaced 1, 5 and 8 days later with normal growth medium and cell survival was assessed on day 9 by the clonogenic assay described in Chapter 2, Materials and Methods, section 2.2.3(d).

The data points represent mean values derived from two flasks. Experiments were run twice at each temperature and best fit regression lines have been fitted to the linear portions of the survival curves using the straight line equation $y = a + bx$.

Correlation coefficients of the best fit regression lines together with D_0 and D_q values, for the higher temperatures are given below.

Temperature ($°C$)	D_0 (mins)	D_q (mins)	Correlation coefficient
43.5	34.0	55	−0.867
44	31.3	33	−0.871
45	18.9	20	−0.927

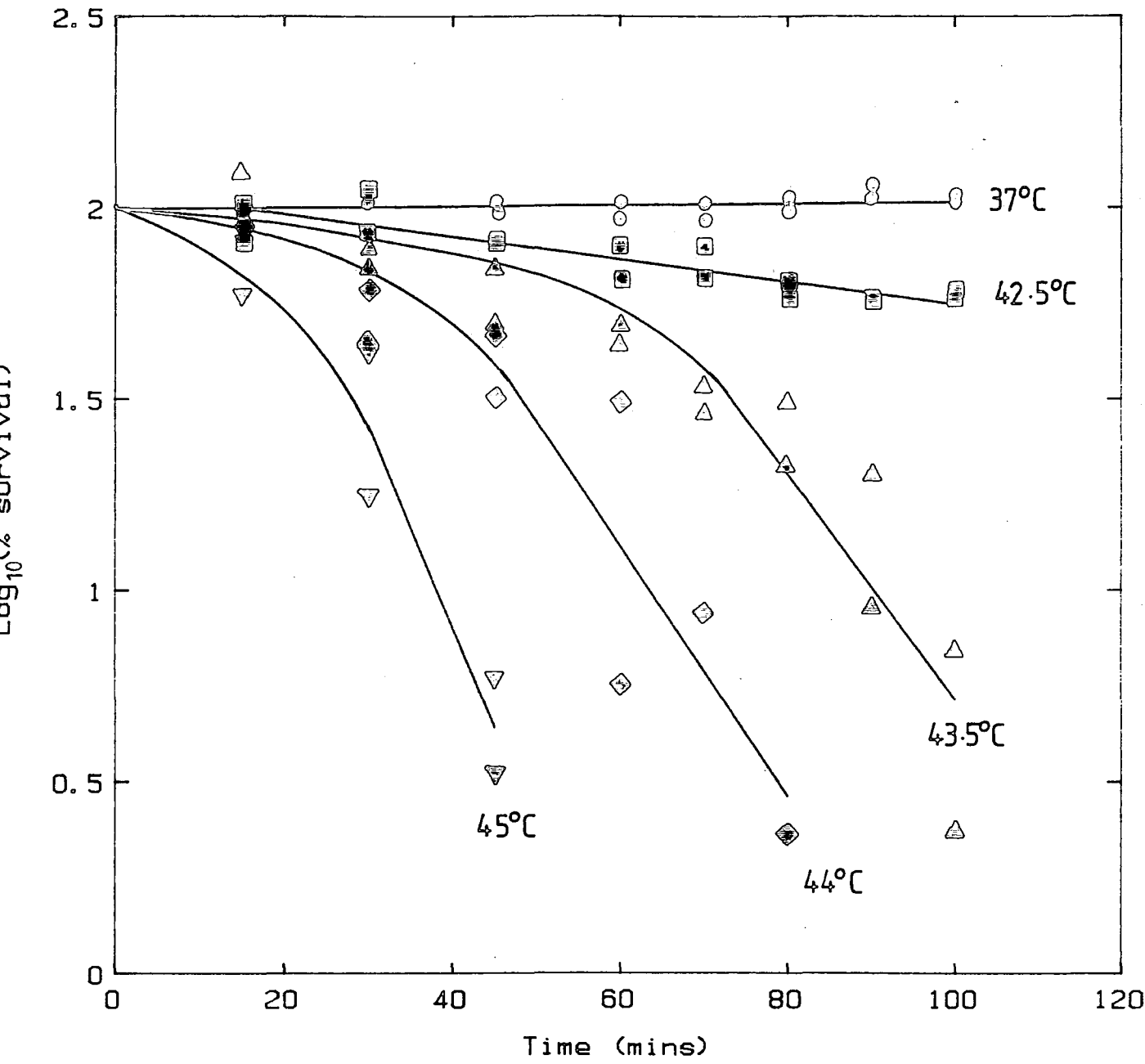

2.5
2
1.5
1
0.5
0
0
20
40
60
80
100
120
Time (mins)
Log_{10}% survival
37°C
42·5°C
43·5°C
44°C
45°C

Figure 3.8

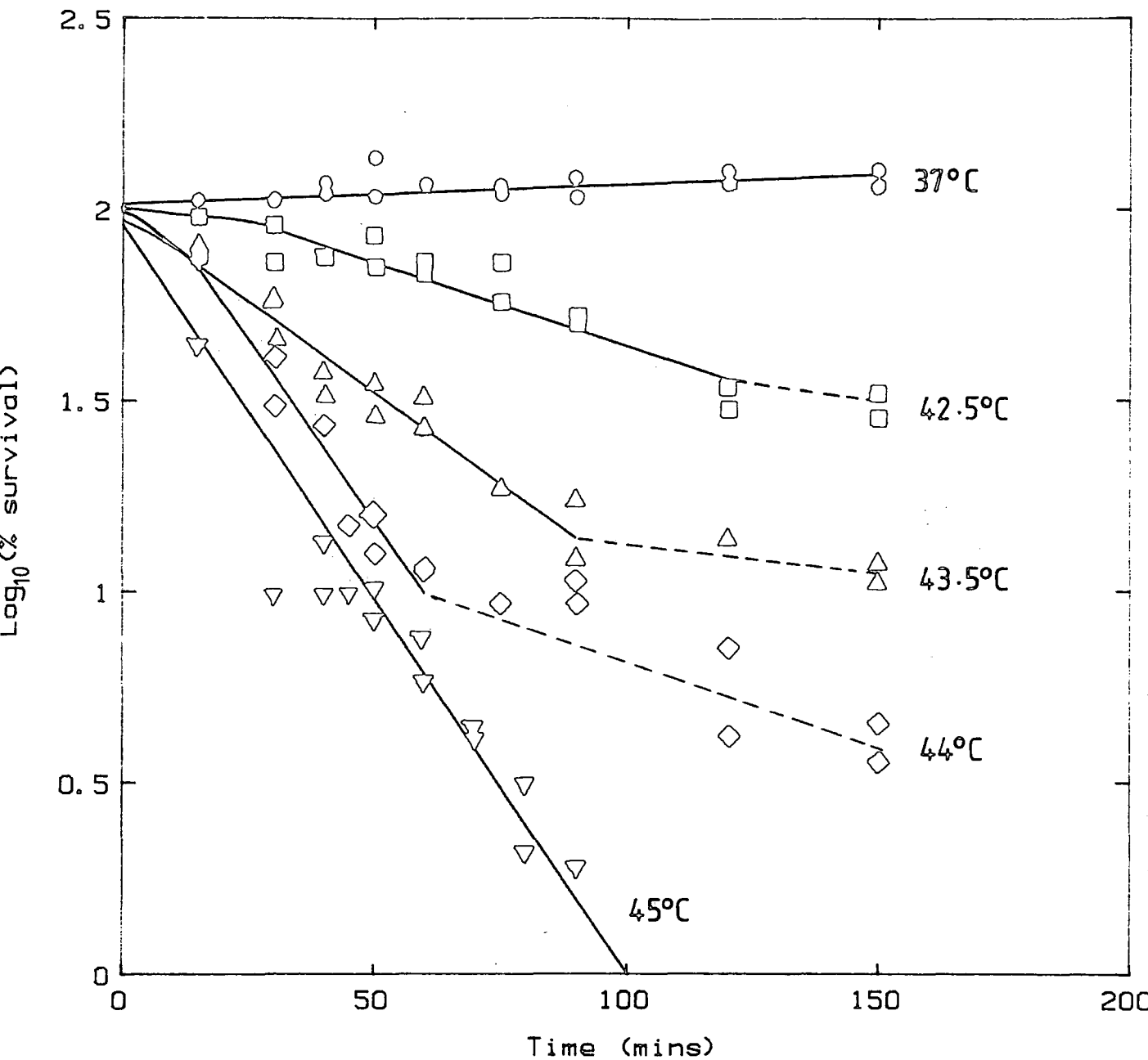
Hyperthermic cell death of HTC cells heated in 24-well plates, assessed by the colorimetric assay

Exponentially growing HTC cells were harvested using EDTA solution and 5×10^3 cells/well were seeded into $1ml$ of growth medium in 24-well plates on day 0 and incubated at $37°C$ under a humid atmosphere of air/CO_2 $(19:1,\ v/v)$. The medium was changed on day 1. On day 3 the medium was replaced with heating medium and after gassing for 20 minutes the plates were sealed and heated as described in Materials and Methods, section 3.2.1(b), at various temperatures ($37°C$ to $45°C$) for various periods of time up to 150 minutes. Following heating, the unsealed plates were returned to the $37°C$ incubator. The medium was changed the following day (day 4) and cell survival was assessed on day 6 by the colorimetric assay described in Chapter 2, Materials and Methods, section 2.2.4(b).

The data points represent mean values derived from 12 wells. Experiments were run twice at each temperature and best fit regression lines have been fitted to the linear portions of the survival curves using the straight line equation $y = a + bx$.

Correlation coefficients of the best fit regression lines together with D_0 and D_q values, for the higher temperatures are given below.

Temperature ($°C$)	D_0 (mins)	D_q (mins)	Correlation coefficient
43.5	105.3	–	−0.968
44	53.2	6	−0.977
45	52.6	–	−0.944


2.5
2
1.5
1
0.5
0
0
50
100
150
200
Log10(% survival)
Time (mins)
37°C
42.5°C
43.5°C
44°C
45°C

into a blue coloured product (formazan). Since this reaction only takes place in living cells utilising the mitochondrial enzyme succinate dehydrogenase, it is thought to reflect the cells' metabolic status.

Considering the different parameters that are being used to measure cell survival and the time after heating that these two assays were performed, it is perhaps not surprising to find that the two assay systems produced substantially different dose response curves. For instance, whilst the clonogenic assay curves (figure 3.7) had marked shoulders over the temperature range $43.5°C$ to $45°C$, these shoulders were very slight or absent in the colorimetric assay (figure 3.8). Similarly, whilst D_0 values in both assay systems showed a decrease with increasing temperature, the D_0 values obtained at $43.5°C$, $44°C$ and $45°C$ of 34, 31.3 and 18.9 minutes in the clonogenic assay were much lower than the corresponding D_0 values of 105.3, 53.2 and 52.6 minutes obtained in the colorimetric assay. In addition, when the survival curves at $42.5°C$, $43.5°C$ and $44°C$ produced by the colorimetric assay are considered, it is evident that unlike the clonogenic assay curves, at longer exposures to these temperatures colorimetric survival curves become biphasic and indicate enhanced cell survival as compared to the clonogenic assay at these time points.

Having characterised the hyperthermic response of HTC cells in terms of the clonogenic and colorimetric assays, a suitable method for presenting fatty acids to cells was developed so that supplementation hyperthermic studies could be undertaken. Attempts at introducing fatty acid into the culture medium by means of celite, a particulate adsorbant (Spector and Hoak, 1969), proved unsuccessful as negligible transfer of fatty acids to serum occurred. However, direct supplementation of serum with fatty acid sodium salts proved a very satisfactory means of supplying fatty acids to cells.

Table 3.2 shows the results of typical free fatty acid assays performed on normal newborn bovine serum (NBS), and NBS that had been supplemented with linoleic acid or stearic acid by the procedure outlined in Materials and Methods, section 3.2.3. It is evident that NBS does contain some endogenous free fatty acid, the level of which varies between batches, and that the supplementation

Table 3.2

Free fatty acid analysis of serum and fatty acid supplemented serum

Three different batches of Newborn Bovine Serum were used to produce fatty acid supplemented serum as described in Materials and Methods, section 3.2.3. The table shows the mean results of typical fatty acid assays run in duplicate on the different batches of serum and their corresponding supplemented products.

Type of serum	Free Fatty Acid concentration (mM)		
	Batch 1	Batch 2	Batch 3
Newborn Bovine Serum (NBS)	0.56	0.53	0.40
NBS-Linoleic Acid	2.11 (E) 1.98 (I)	2.03 (E) 1.93 (I)	1.98 (E) 1.97 (I)
NBS-Stearic Acid	2.24 (E) 2.18 (I)	2.00 (E) 1.67 (I)	2.04 (E) 1.73 (I)

E = Expected I = Increase due to supplementation

procedure finally adopted consistently produced stock solutions with fatty acid concentrations close to the desired $2mM$ figure.

Figures 3.9 and 3.10 show the effect of exposing exponentially growing HTC cells to increasing concentrations of either linoleic acid (18 : 2) or stearic acid (18 : 0) supplemented growth medium for a 24 hour or 48 hour period. In figure 3.9 the effect of a 48 hour period of supplementation with various concentrations of the two fatty acids in growth medium containing 5% (v/v) NBS are shown. Linoleic acid at all concentrations served to increase cell number relative to control cells with the increase in cell number showing a positive correlation with fatty acid concentration suggesting a stimulatory effect of linoleic acid on cell growth. In the case of stearic acid, whilst concentrations up to $20\mu M$ served to produce an increase in cell number relative to control cells, at higher concentrations (40-$60\mu M$) there was a marked reduction in cell number relative to control cells suggesting an inhibitory effect on cell growth.

In figure 3.10 the effect of both 24 hour and 48 hour periods of fatty acid supplementation with slightly different concentrations of the two fatty acids, in growth medium containing 10% (v/v) NBS are shown. In the case of linoleic acid, the same trend in effect is visible at 24 hours and 48 hours and reflects the results seen in medium containing 5% (v/v) NBS, that is, increasing concentrations up to $60\mu M$ linoleic acid serve to increase the number of cells relative to control cells. At $80\mu M$ linoleic acid concentrations, the apparent stimulatory effect of this fatty acid is abolished and cell number more closely reflects that of control cells. In the case of stearic acid it can be seen that even when very low concentrations were used, cell number relative to control cells showed a decrease with time again suggesting an inhibitory effect of this saturated fatty acid on cell growth.

The supplemented concentrations of fatty acid to be used in hyperthermic studies were those that did not interfere with cell growth in a marked fashion prior to hyperthermic treatment. Figures 3.11, 3.12 and 3.13 show typical growth curves produced in flasks and plates where cells had been grown and supplemented in growth medium containing either 5% (v/v) or 10% (v/v) NBS and exposed to various concentrations of fatty acid supplement over a two day period.

Figure 3.9

The effect of supplemental fatty acid concentration on cell number in the presence of growth medium containing reduced levels of serum

Exponentially growing HTC cells that had been grown for several passages in growth medium containing 5% (v/v) newborn bovine serum (5% growth medium) were harvested using EDTA solution and 3×10^5 cells were seeded into $10ml$ of 5% growth medium, in $25cm^2$ flasks on day 0 and incubated at $37°C$ under a humid atmosphere of air/CO_2 (19 : 1, v/v). The 5% growth medium was changed the following day. On day 2 cells were exposed to 5% growth medium supplemented with either linoleic acid or stearic acid at concentrations ranging from 0 to $60\mu M$ for a 48 hour period. Control cells were maintained in 5% growth medium at all times. The 5% growth medium of control cells and the supplemented 5% growth medium were replaced after the first 24 hours. The number of cells present in each flask after the 48 hour period was determined using a Coulter Counter.

All points, except the control value, represent individual results from a single experiment. The control value shown represents the mean value from two flasks.

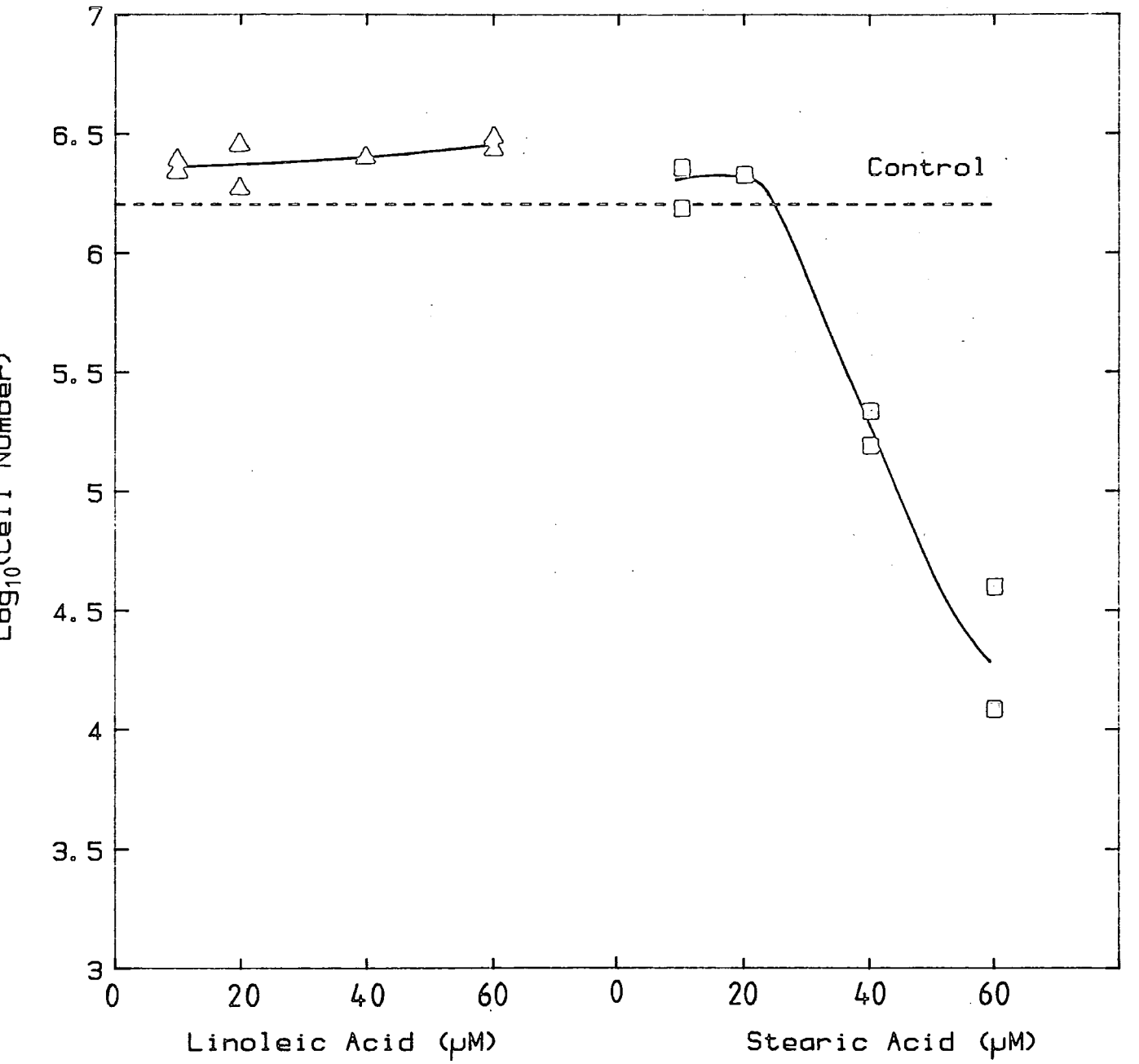
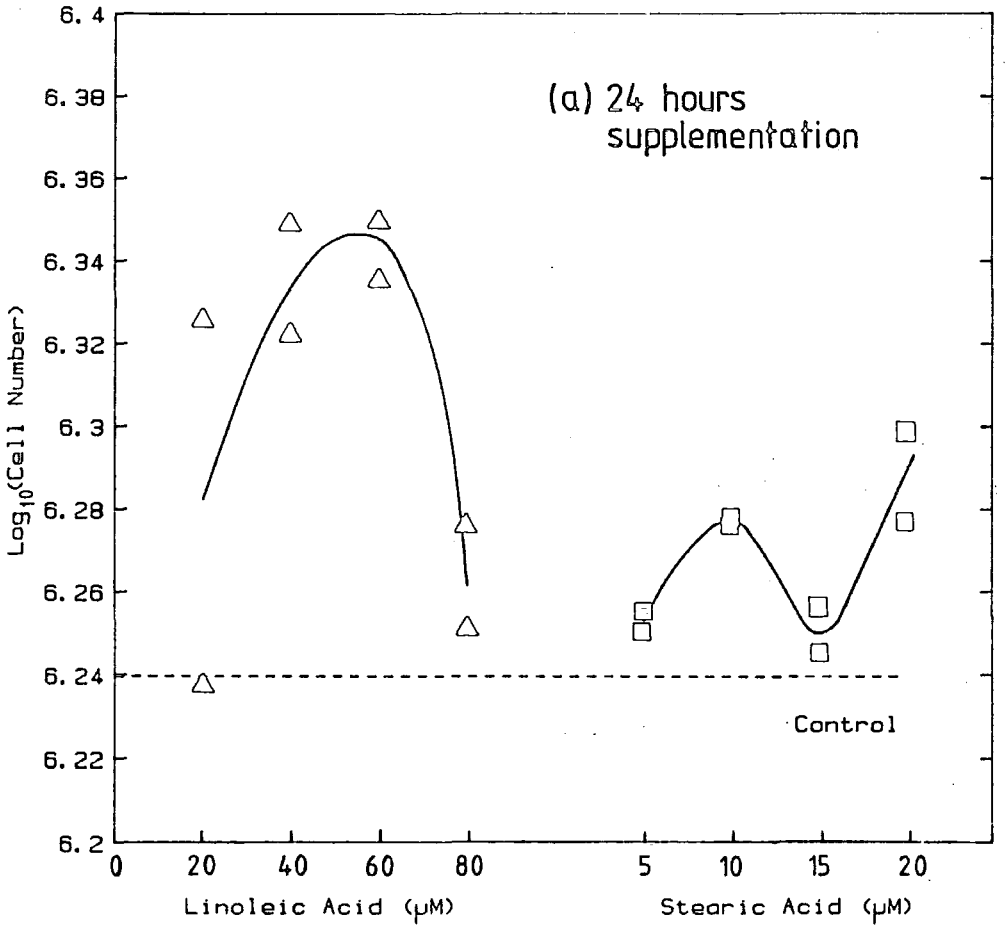

7
6.5
6
5.5
5
4.5
4
3.5
3
Log10(Cell Number)
Control
0
20
40
60
0
20
40
60
Linoleic Acid (μM)
Stearic Acid (μM)

Figure 3.10

The effect of supplemental fatty acid concentration on cell number in the presence of normal growth medium

Exponentially growing HTC cells were harvested using EDTA solution and 3×10^5 cells/$25cm^2$ flask were seeded into $10ml$ of growth medium containing 10% (v/v) NBS i.e. normal growth medium, on day 0 and incubated at $37°C$ under a humid atmosphere of air/CO_2 ($19:1$, v/v). The medium was changed the following day. On day 2 cells were exposed to normal growth medium supplemented with either linoleic acid or stearic acid at concentrations ranging from 0 to $80\mu M$ and 0 to $20\mu M$ respectively for 24 and 48 hour periods. Control cells were maintained in normal growth medium at all times. The normal growth medium of control cells and the supplemented medium were replaced after the first 24 hours. The number of cells present in each flask after the 24 and 48 hour periods was determined using a Coulter Counter.

All points, except control values, represent individual results of a single experiment. The control values shown represent the mean values derived from two flasks.


(a) 24 hours
supplementation
Log10(Cell Number)
6.4
6.38
6.36
6.34
6.32
6.3
6.28
6.26
6.24
6.22
6.2
Control
0
20
40
60
80
5
10
15
20
Linoleic Acid (μM)
Stearic Acid (μM)

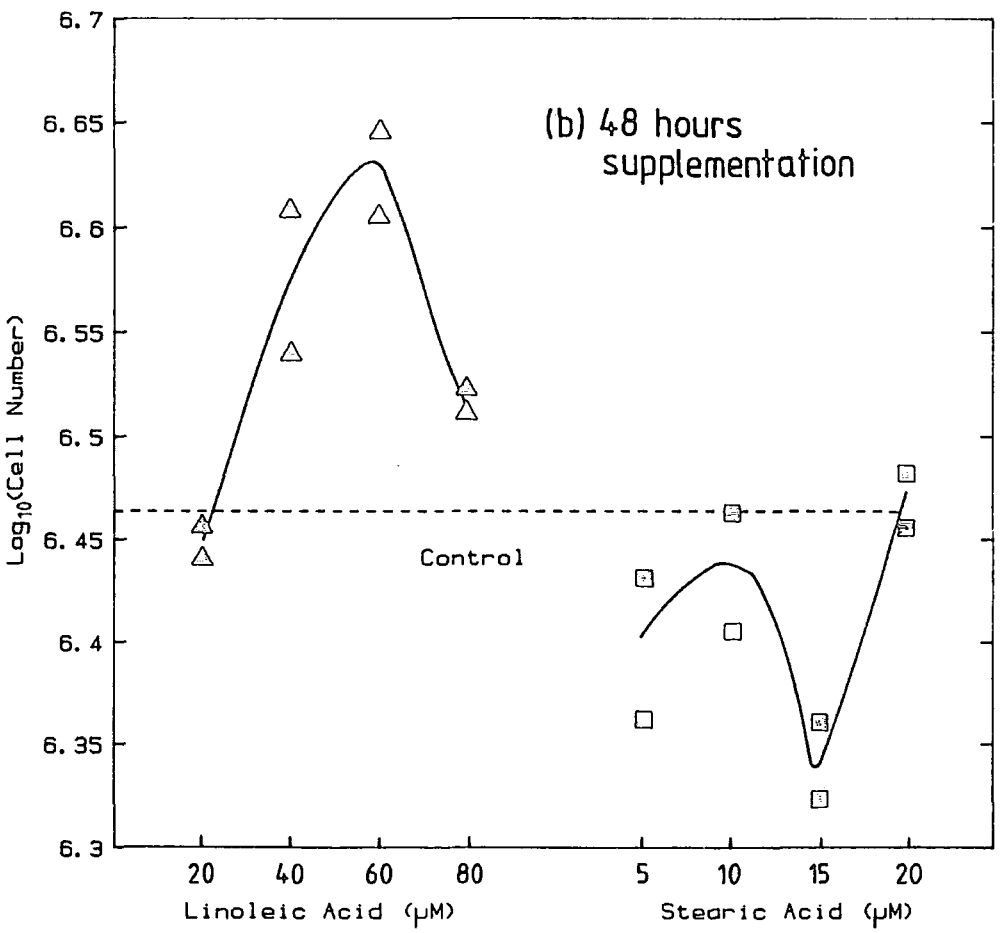

(b) 48 hours
supplementation
Log10(Cell Number)
6.7
6.65
6.6
6.55
6.5
6.45
6.4
6.35
6.3
Control
20
40
60
80
5
10
15
20
Linoleic Acid (μM)
Stearic Acid (μM)

Figure 3.11

Growth curves of HTC cells grown and supplemented in flasks in the presence of growth medium containing reduced levels of serum

Flasks ($25cm^2$) were seeded with 2.5×10^5 cells in $10ml$ of growth medium containing 5% (v/v) newborn bovine serum (5% growth medium) on day 0 and incubated at $37°C$ under a humid atmosphere of air/CO_2 (19 : 1, v/v). The medium was changed on day 1. On day 2 cells were exposed either to $20\mu M$ stearic acid or $40\mu M$ linoleic acid in $10ml$ of 5% growth medium for a 48 hour period, with a media change after the first 24 hours. Cell number was then determined by the Coulter Counter method described in Chapter 2, Materials and Methods, section 2.2.3(c).

Each point on the graph represents the mean value derived from a total of 4 flasks run in two separate experiments. The standard errors of flask data are shown below.

Day	Cell number $\times 10^{-5}$		
	5% growth medium	5% growth medium $+40\mu M$ linoleic acid	5% growth medium $+20\mu M$ stearic acid
0	2.5	2.5	2.5
1	3.50 ± 0.13	3.50 ± 0.13	3.50 ± 0.13
2	3.24 ± 0.53	3.24 ± 0.13	3.24 ± 0.13
3	10.87 ± 2.62	11.74 ± 3.14	12.56 ± 3.66
4	25.73 ± 4.28	26.27 ± 11.01	8.90 ± 5.33

Results represent mean value $\pm S.E.M.$ ($n = 4$).

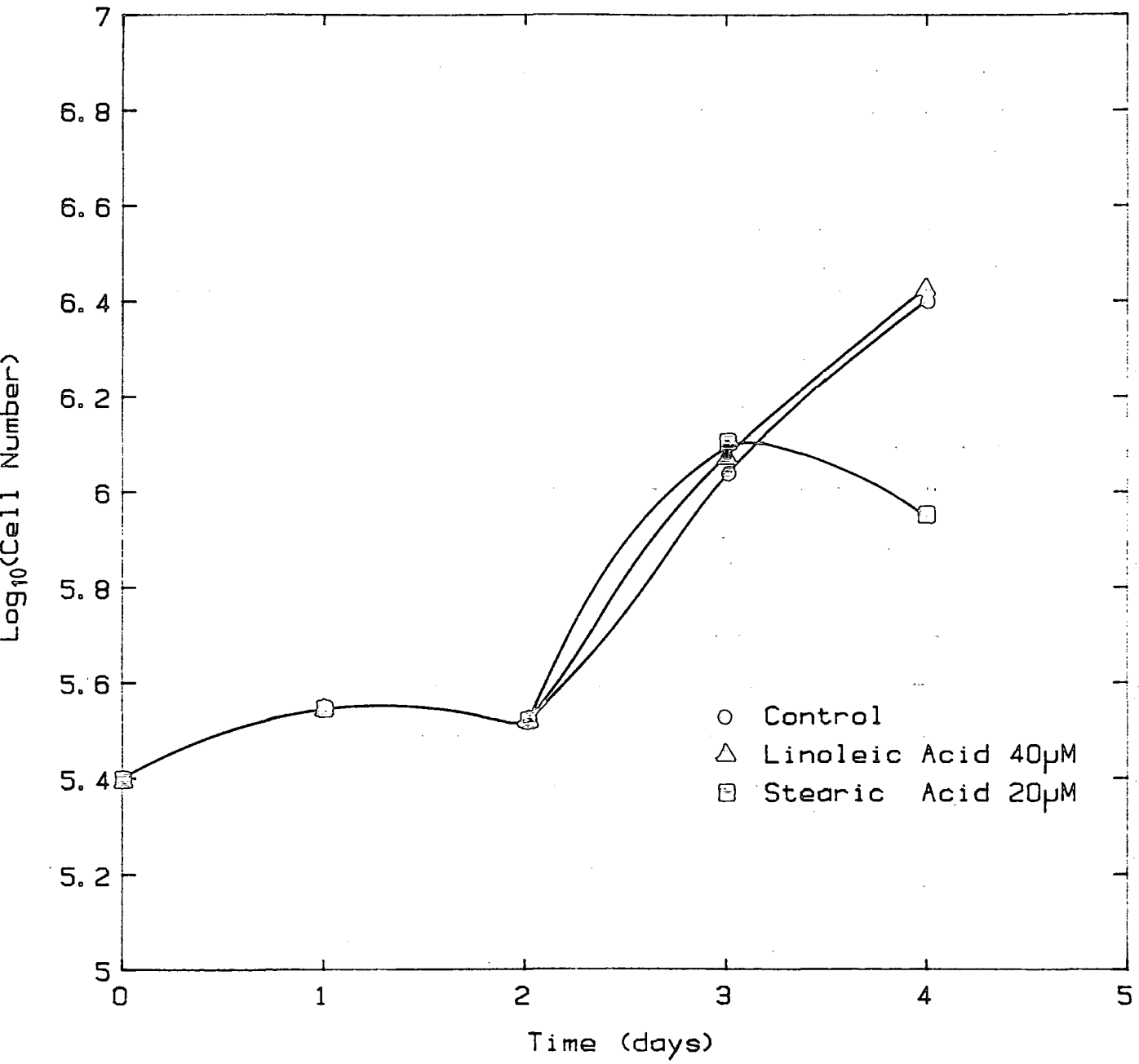

7
6.8
6.6
6.4
6.2
6
5.8
5.6
5.4
5.2
5
0
1
2
3
4
5
Log10(Cell Number)
Time (days)
Control
Linoleic Acid 40μM
Stearic Acid 20μM

Figure 3.12

Growth curves of HTC cells in flasks in the presence of normal growth medium

Flasks were seeded with 3.0×10^5 cells in $10ml$ of growth medium containing 10% (v/v) NBS (normal growth medium) on day 0 and incubated at $37°C$ under a humid atmosphere of air/CO_2 $(19:1,\ v/v)$. The medium was changed on day 1. On day 2 cells were exposed to normal growth medium supplemented with a range of linoleic acid concentrations (40 to $60\mu M$) for a 48 hour period. Control cells were maintained in normal growth medium at all times. The normal growth medium and supplemented medium were replaced after the first 24 hours. Cell number was determined by the Coulter Counter method described in Chapter 2, Materials and Methods, section 2.2.3(c).

Each point on the graph represents the mean value derived from a total of 4 flasks run in two separate experiments. The standard errors of flask data are shown below.

Day	Cell number $\times 10^{-5}$			
	Normal growth medium	Normal growth medium + $40\mu M$ linoleic acid	Normal growth medium + $60\mu M$ linoleic acid	Normal growth medium + $80\mu M$ linoleic acid
0	3.0	3.0	3.0	3.0
1	2.84 ± 0.24	2.84 ± 0.24	2.84 ± 0.24	2.84 ± 0.24
2	7.20 ± 0.63	7.20 ± 0.63	7.20 ± 0.63	7.20 ± 0.63
3	16.30 ± 0.73	18.65 ± 1.75	18.00 ± 2.59	15.76 ± 1.55
4	28.18 ± 0.93	33.15 ± 2.79	34.26 ± 4.64	28.20 ± 2.83

Results represent mean value $\pm S.E.M.$ $(n = 4)$.

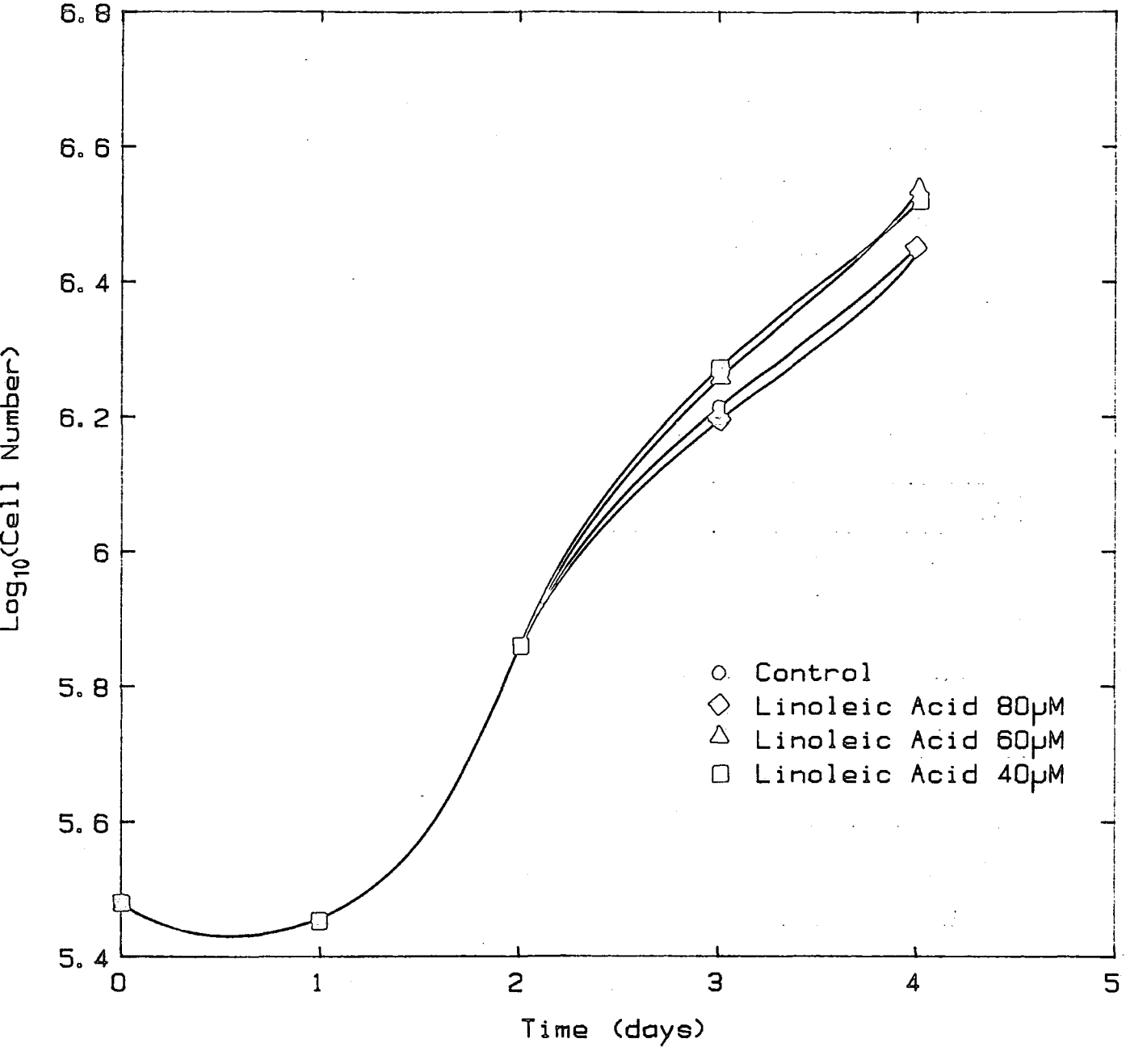
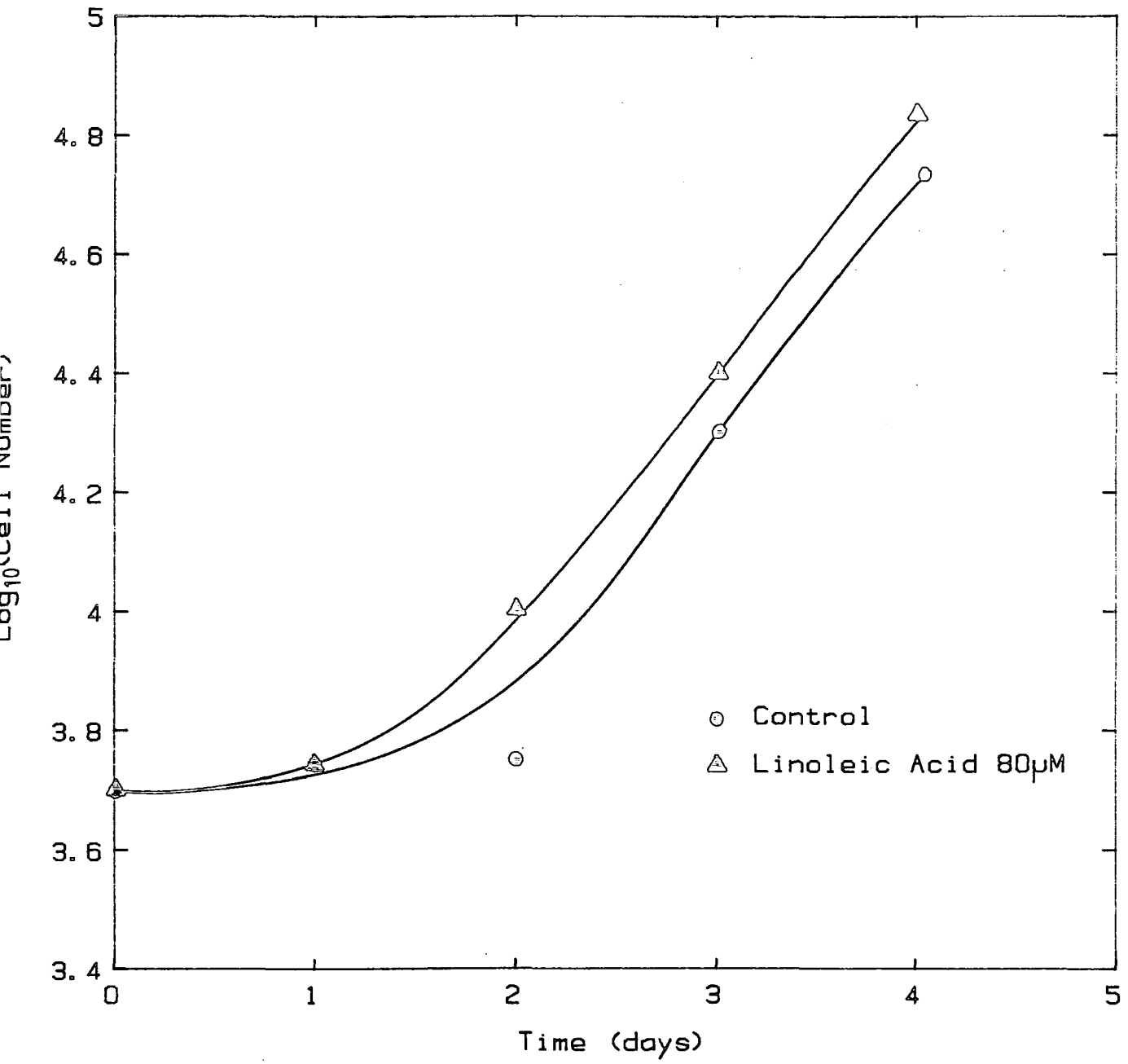

6.8
6.6
6.4
6.2
6
5.8
5.6
5.4
Log10(Cell Number)
0
1
2
3
4
5
Time (days)
Control
Linoleic Acid 80μM
Linoleic Acid 60μM
Linoleic Acid 40μM

Figure 3.13

Growth curves of HTC cells grown and supplemented in 24-well plates in the presence of normal growth medium

Plates (24-well) were seeded with 5×10^3 cells/well in $1ml$ of growth medium containing 10% (v/v) NBS (normal growth medium) on day 0 and incubated at $37°C$ under a humid atmosphere of air/CO_2 (19 : 1, v/v). On day 1 the cells were exposed to normal growth medium containing $80\mu M$ linoleic acid supplement for a 72 hour period. Control cells were maintained in normal growth medium at all times. The normal growth medium and supplemented medium were replaced after 24 and 48 hours. The number of cells present in wells over the 4 day period was determined by the modified Mosmann method described in Chapter 2, Materials and Methods, section 2.2.4(b). Cell numbers were estimated from figure 2.8 of the same chapter. Cell number estimates shown on the graph are based on mean absorbance values derived from 4 replicate wells in a single experiment.


5
4.8
4.6
4.4
4.2
4
3.8
3.6
3.4
0
1
2
3
4
5
Log10(Cell Number)
Time (days)
Control
Linoleic Acid 80μM

From figure 3.11, where cells were grown and supplemented in flasks in growth medium containing 5% (v/v) NBS, it is evident that stearic acid has an inhibitory effect on cell growth at a relatively low concentration. Because growth of HTC cells in the presence of this saturated fatty acid was adversely affected under routine culture conditions it was not utilised in subsequent hyperthermic studies. Interest came to focus on linoleic acid which even at concentrations as high as $80\mu M$ did not have an inhibitory effect on cell growth.

Figure 3.12 shows typical growth curves produced in flasks in the presence and absence of a range of linoleic acid concentrations, when cells were grown and supplemented in growth medium containing 10% (v/v) NBS. Figure 3.13 shows typical growth curves produced in plates in the presence and absence of $80\mu M$ linoleic acid when the cells were exposed to supplement the day after seeding rather than two days after seeding. In both cases it would appear that the growth of cells is stimulated slightly in the presence of the supplemented fatty acid.

In figure 3.14, the photomicrographs (a) to (e) show the effect of $80\mu M$ linoleic acid supplementation on cytoplasmic lipid droplet formation in HTC cells grown on coverslips in 24-well plates. It can be seen that droplets are formed in the cytoplasm within 6 hours of supplementation (compare figure 3.14(a) with figure 3.14(b)) and that the numbers of droplets increases as the length of the supplementation period increases up to 72 hours (figures (c), (d) and (e)). The lipid droplets appear to concentrate at the periphery of cells (figure (e)). Figure 3.14(f) shows the appearance of control cells at the end of the 72 hour period which contrasts markedly to that of supplemented cells (figure 3.14(e)) clearly indicating that the difference in appearance of the cells is due to the presence of supplement.

Figures 3.14(g) and (h) demonstrate the effect of returning cells that have been exposed to $80\mu M$ linoleic acid supplemented growth medium for various periods of time to normal medium. The cells in figure 3.14(g) were exposed to linoleic acid supplement for a 24 hour period and were then returned to normal growth medium for a 72 hour period before being examined. It is evident that the vacuoles generated during the supplementation period (figure 3.14(c)) have

Figure 3.14

The effect of fatty acid supplementation on cytoplasmic lipid droplet formation

Plates (24-well) were seeded with 5×10^3 cells/well in $1ml$ of growth medium containing 10% (v/v) NBS (normal growth medium) into wells containing glass coverslips on day 0 and maintained in an incubator at $37°C$ under a humid atmosphere of air/CO_2 (19 : 1, v/v). The medium was changed on day 1. On day 2 the cells were exposed to normal growth medium containing $80\mu M$ linoleic acid supplement for various periods of time as described below. Control cells were maintained in normal growth medium at all times. The normal growth medium and supplemented medium were replaced every 24 hours. Photographs were taken with a Nikon camera using an inverted Nikon Diaphot microscope fitted with Normarski optics.

(a) Control HTC cells at the 6 hour supplementation time point.

(b) HTC cells 6 hours after supplementation.

(c) HTC cells 24 hours after supplementation.

(d) HTC cells 48 hours after supplementation.

(e) HTC cells 72 hours after supplementation.

(f) Control HTC cells at the 72 hour supplementation time point.

(g) HTC cells supplemented for 24 hours and then returned to normal medium for 72 hours.

(h) HTC cells supplemented for 72 hours and then returned to normal medium for 24 hours.

All photomicrographs ×280.

a

b

c

d

e

f

g

h

disappeared. In the case of figure 3.14(h) the cells were exposed to supplement for a 72 hour period and then returned to normal growth medium for a 24 hour period before being examined. It can be seen that whilst vacuoles are still present they are greatly reduced in number as compared to the number present immediately after a 72 hour supplementation period (figure 3.14(e)).

Overall, the photomicrographs suggest that when HTC cells are exposed to the unsaturated fatty acid, linoleic acid in the growth medium it is taken up very rapidly into the cell and accumulates to form cytoplasmic droplets, the number of which increases with time. They also indicate that these droplets quickly disappear when cells are returned to normal growth medium.

In the supplementation hyperthermic studies that were conducted, two temperatures of $43°C$ and $43.5°C$ were selected for use since they were known to produce reasonable rates of cell kill over the experimental time courses that were planned. In the case of flask experiments, the time required to kill 90% of the cells (LD_{90}) at a particular temperature has been used as an index of cytotoxicity since this value occurs on the linear portion of the survival curves and reflects the effect of both the shoulder region and the slope of the curve (Bhuyan, 1979). In the case of supplementation hyperthermia experiments conducted in plates, because a wider range of temperatures were investigated and because of the biphasic nature of the curves shown earlier (figure 3.8), LD_{50} values which occur in the first linear region of the survival curves have been quoted as an index of cytotoxicty.

Figures 3.15 and 3.16 show the results of hyperthermic studies on control and fatty acid supplemented cells, conducted in flasks at $43.5°C$ over a 100 minute time period. When cells were supplemented with $40\mu M$ linoleic acid for a 48 hour period (figure 3.15) prior to heating there was no significant effect on the hyperthermic survival curves compared to control cells. The D_0 values for control and supplemented cells were 38.8 and 43.1 minutes respectively, whilst the LD_{90} values were 88 and 90 minutes respectively. When the concentration of linoleic acid in the growth medium was increased to $60\mu M$ (figure 3.16(a) and (b)), the effect of both a 24 hour period of supplementation (figure 3.16(a)) and a 36 hour period of supplementation (figure 3.16(b)) prior to heating at $43.5°C$ was

Figure 3.15

The effect of a 48 hour period of supplementation with 40μM linoleic acid on the thermal sensitivity of HTC cells exposed to 43.5°C in flasks

Cells (3×10^5 cells/25cm^2 flask) were seeded into 10ml of normal growth medium on day 0 and incubated at 37°C under a humid atmosphere of air/CO_2 (19 : 1, v/v). The medium was changed on day 1. On day 2 cells were exposed to normal growth medium supplemented with 40μM linoleic acid. Control cells were maintained in normal growth medium. The normal growth medium and supplemented medium were replaced after 24 hours (day 3). After 48 hours (day 4) the exponentially growing cells were harvested using EDTA solution and 200 cells/25cm^2 flask were seeded into 10ml of heating medium and returned to the 37°C incubator for 3 hours. Flasks were then sealed and heated at 43.5°C for various periods of time up to 100 minutes. Following heating, unsealed flasks were returned to the 37°C incubator. The medium was replaced 1, 5 and 8 days later with normal growth medium and cell survival was assessed on day 9 by the clonogenic assay described in Chapter 2, Materials and Methods, section 2.2.3(d).

Individual results of a single experiment run in duplicate are shown on the graph. Best fit regression lines have been fitted to the linear portions of the survival curves using the straight line equation $y = a + bx$.

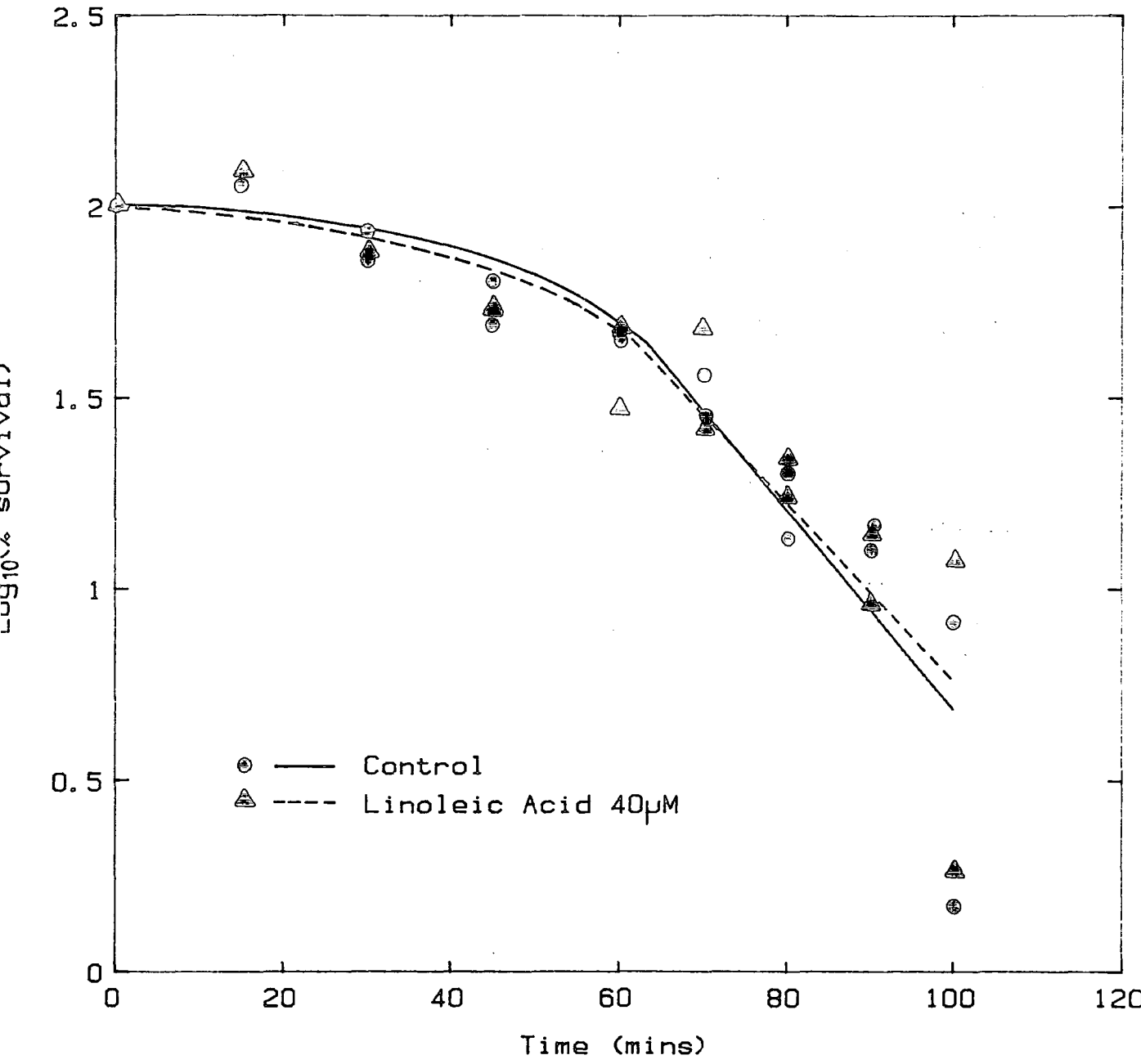

2.5
2
1.5
1
0.5
0
0
20
40
60
80
100
120
Time (mins)
Control
Linoleic Acid 40μM

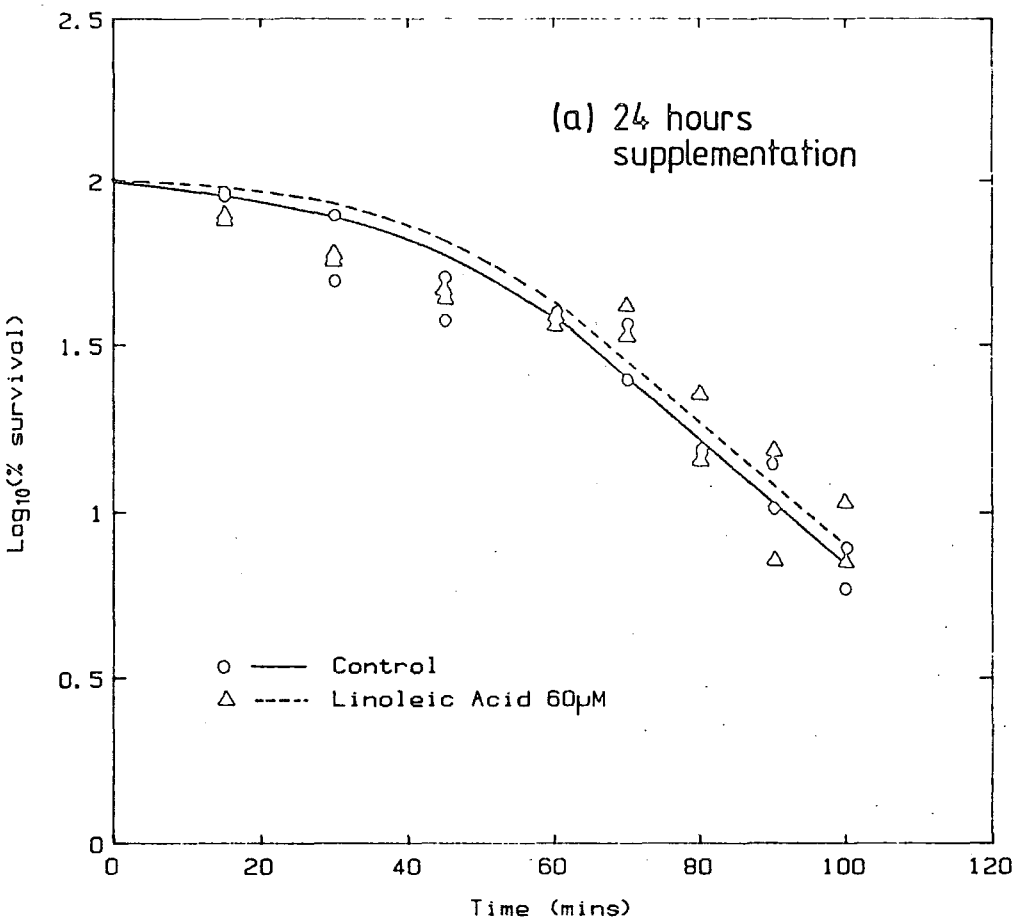
Figure 3.16

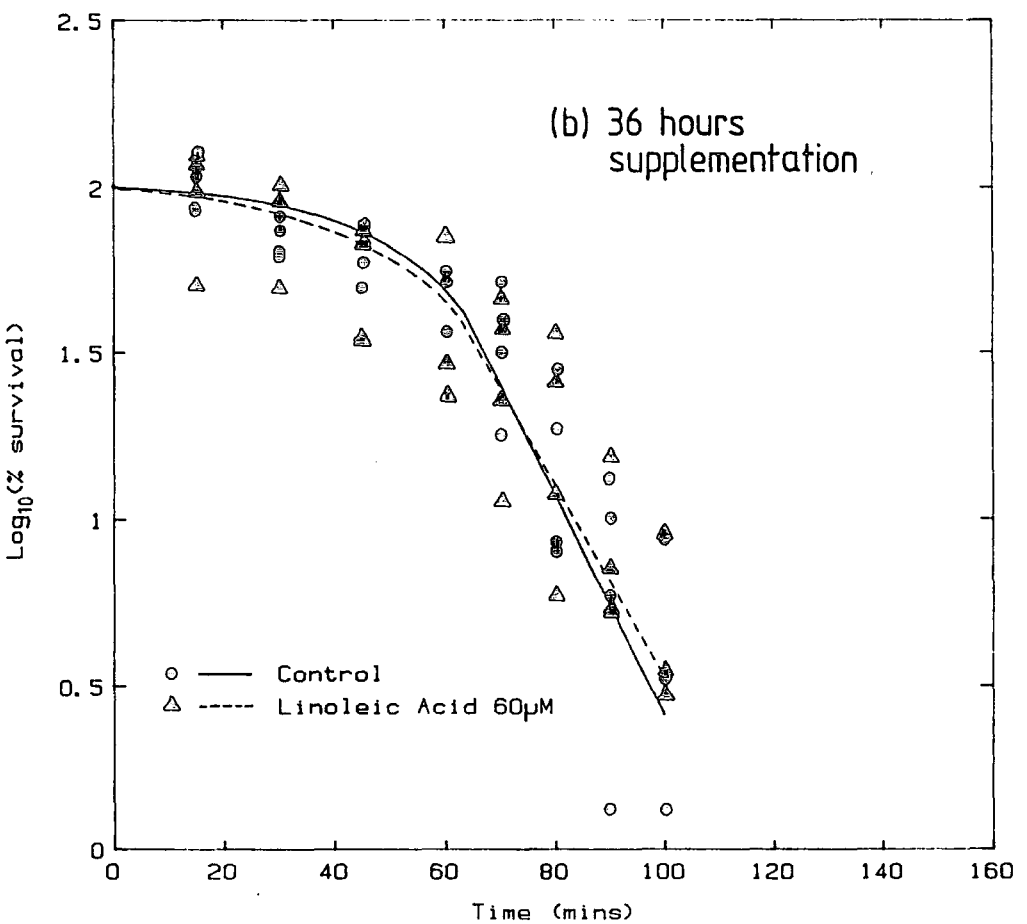
The effect of 24 hour and 36 hour periods of supplementation with 60μM linoleic acid on the thermal sensitivity of HTC cells exposed to 43.5°C in flasks

Cells (3×10^5 cells/$25cm^2$ flask) were seeded into $10ml$ of normal growth medium on day 0 and incubated at $37°C$ under a humid atmosphere of air/CO_2 ($19:1$, v/v). The medium was changed on day 1. Cells were exposed to normal growth medium supplemented with $60\mu M$ linoleic acid at appropriate times on day 2 and day 3 to achieve 24 hour and 36 hour periods of supplementation by day 4. Control cells were maintained in normal growth medium. On day 4 cells were harvested and treated as described in the legend of figure 3.15.

Figure 3.16(a) shows individual results of a single experiment run in duplicate. Figure 3.16(b) shows individual results from two experiments run in duplicate.

Best fit regression lines have been fitted to the linear portions of the survival curves using the straight line equation $y = a + bx$.


(a) 24 hours
supplementation
Control
Linoleic Acid 60μM
Log10(% survival)
Time (mins)
2.5
2
1.5
1
0.5
0
0
20
40
60
80
100
120


(b) 36 hours
supplementation
Control
Linoleic Acid 60μM
Log10(% survival)
Time (mins)
2.5
2
1.5
1
0.5
0
0
20
40
60
80
100
120
140
160

again minimal. After 24 hours the D_0 values for control and supplemented cells were 53.7 and 54.6 minutes respectively, whilst the LD_{90} values were 91.5 and 94.5 minutes respectively suggesting a slight, though not significant ($p > 0.05$), decrease in thermal sensitivity of supplemented cells. Following a 36 hour period of supplementation prior to heating, the D_0 values for control and supplemented cells were 30.3 and 34.5 minutes respectively, whilst the LD_{90} values were 82 and 83 minutes (figure 3.16(b)).

Longer periods of heating at the slightly lower temperature of $43°C$ were then utilised, again in flasks, and the results of 24, 36 and 48 hour periods of supplementation with $60\mu M$ linoleic acid prior to heat treatment are shown in figures 3.17, 3.18 and 3.19. Figure 3.17 shows the survival curves obtained in flasks following a 24 hour period of supplementation with $60\mu M$ linoleic acid prior to heat treatment. It is evident that there is a high level of variability in the data presented in the figure. Consequently, the D_0 values of 125.8 and 96.2 minutes for control and supplemented cells and the LD_{90} values of 165 and 152 minutes respectively that have been deduced from the best fit regression lines can not be taken as being significantly different.

However, the data shown in figure 3.18 is much more consistent. Regression lines fitted to the linear portion of the survival curves (with regression correlation coefficients, $|r| > 0.94$) indicate that a 36 hour period of supplementation with $60\mu M$ linoleic acid prior to exposure to $43°C$ produces a significant increase in the thermal sensitivity of HTC cells in terms of LD_{90} values as determined by the clonogenic assay. Whilst there is a slight change in the D_0 values from 53.2 to 52.4 minutes respectively for control and supplemented cells, it can be seen that the LD_{90} value is reduced from 139.8 minutes in the case of control cells to 130 minutes in the case of supplemented cells, with these two values being significantly different at the $p = 0.05$ level. The fact that cell survival is consistently reduced throughout the linear portion of the curve strongly suggests that supplementation of HTC cells for a 36 hour period with $60\mu M$ linoleic acid prior to heating increases their thermal sensitivity.

Following a 48 hour period of supplementation with $60\mu M$ linoleic acid (figure 3.19) this significant increase in thermal sensitivity in terms of LD_{90} values is

Figure 3.17

The effect of a 24 hour period of supplementation with 60μM linoleic acid on the thermal sensitivity of HTC cells exposed to 43°C in flasks

Cells (3×10^5 cells/$25cm^2$ flask) were seeded into $10ml$ of normal growth medium on day 0 and incubated at $37°C$ under a humid atmosphere of air/CO_2 (19 : 1, v/v). The medium was changed on day 1. Cells were exposed to normal growth medium supplemented with $60\mu M$ linoleic acid on day 3. Control cells were maintained in normal growth medium. 24 hours later (day 4) the exponentially growing cells were harvested and 200 cells/$25cm^2$ flask were seeded into $10ml$ of heating medium and returned to the $37°C$ incubator for 3 hours. Flasks were then sealed and heated at $43°C$ for various periods of time up to 210 minutes. Post heating treatment was as described in the legend of figure 3.15.

Individual results from two experiments run in duplicate are shown on the graph.

Best fit regression lines have been fitted to the linear portions of the survival curves using the straight line equation $y = a + bx$.

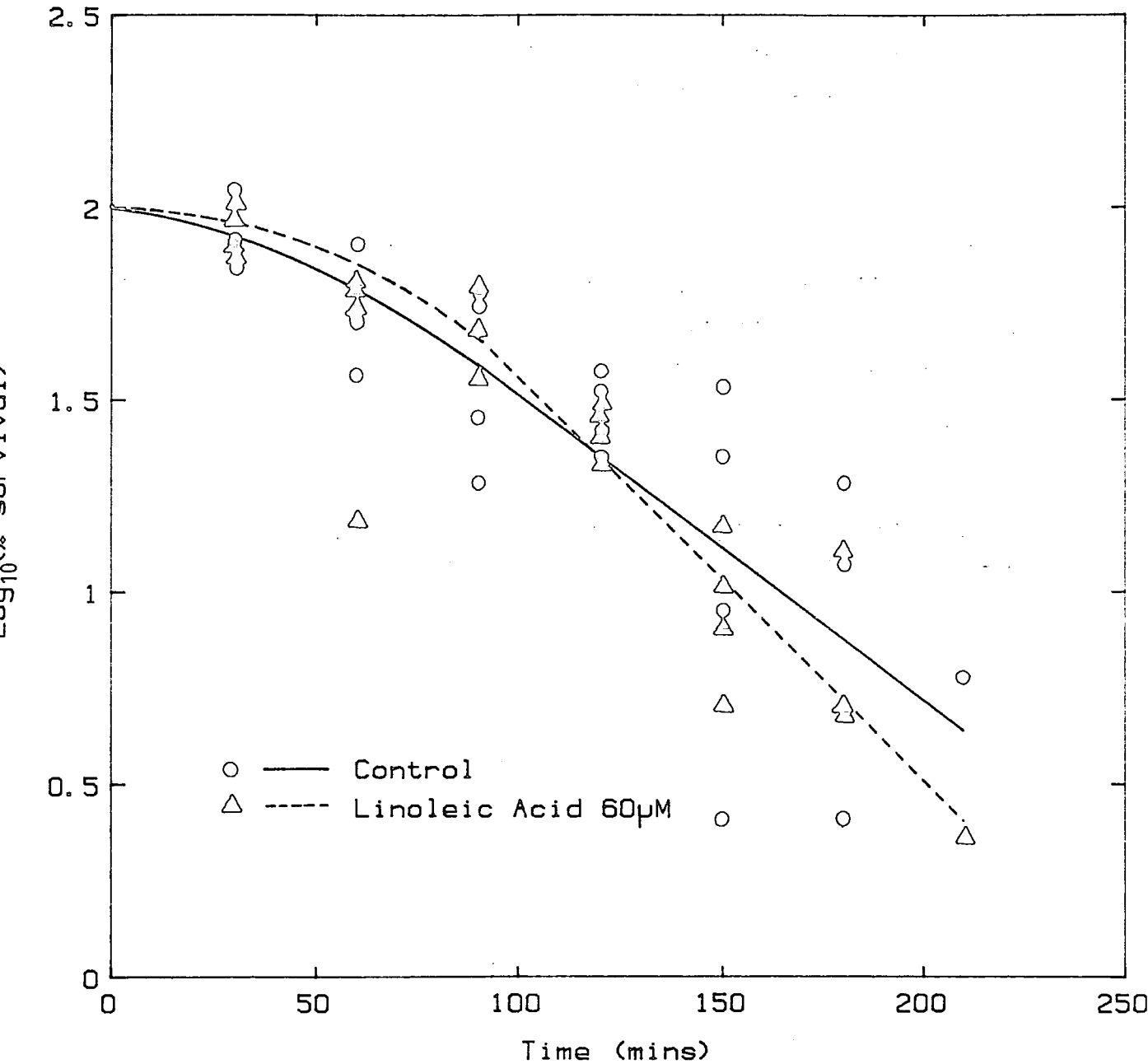

2.5
2
1.5
1
0.5
0
0
50
100
150
200
250
Time (mins)
Control
Linoleic Acid 60μM

Figure 3.18

The effect of a 36 hour period of supplementation with 60μM linoleic acid on the thermal sensitivity of HTC cells exposed to 43°C in flasks

Experimental details are as described in the legend of figure 3.17 except that cells were exposed to normal growth medium supplemented with 60μM linoleic acid for a 36 hour period prior to heating rather than a 24 hour period.

Individual results from two experiments run in duplicate are shown on the graph.

Best fit regression lines have been fitted to the linear portions of the survival curves using the straight line equation $y = a + bx$.

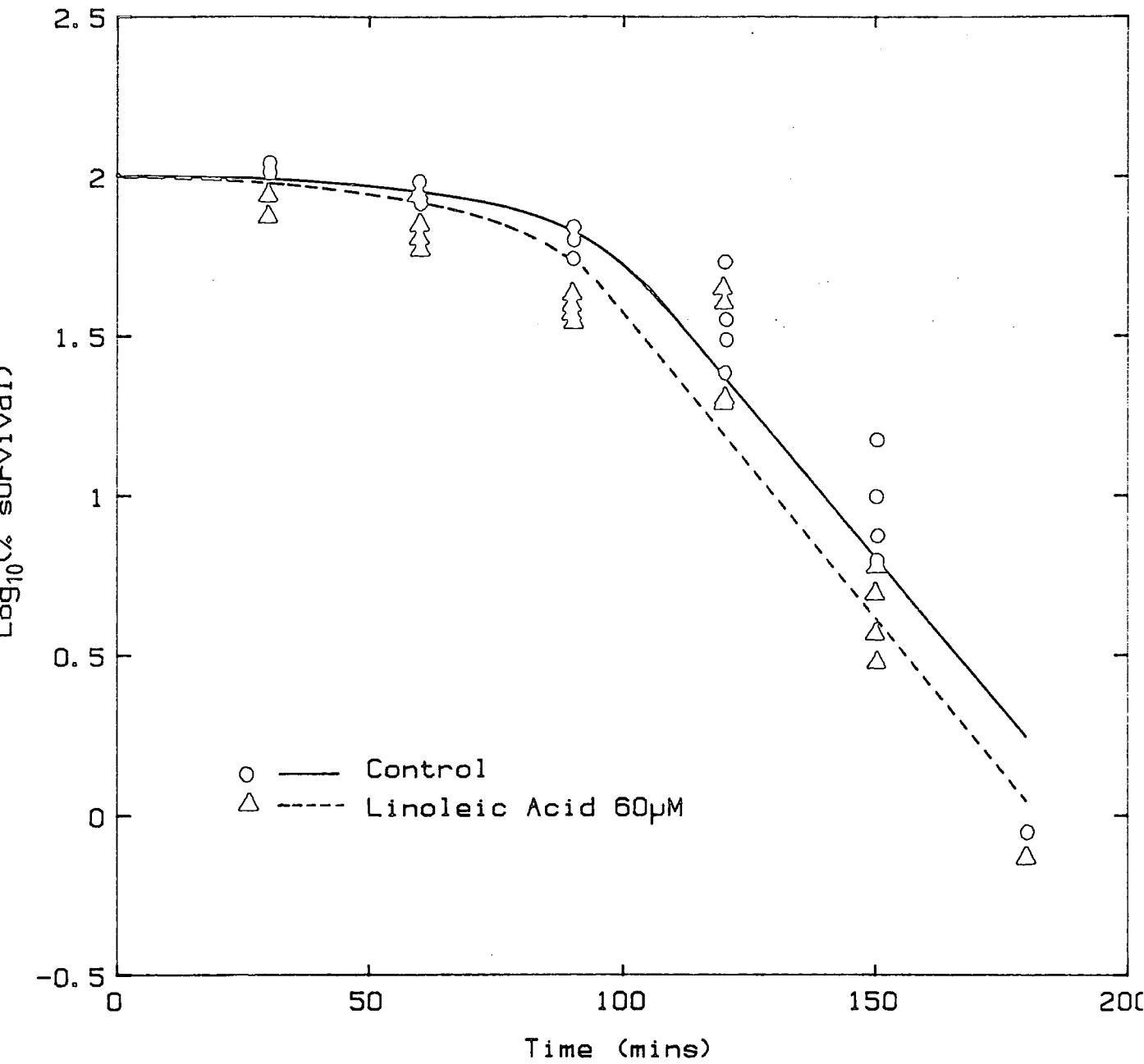

2.5
2
1.5
1
0.5
0
-0.5
0
50
100
150
200
Log10(% survival)
Time (mins)
Control
Linoleic Acid 60μM

Figure 3.19

The effect of a 48 hour period of supplementation with $60\mu M$ linoleic acid on the thermal sensitivity of HTC cells exposed to $43°C$ in flasks

Experimental details are as described in the legend of figure 3.17 except that cells were exposed to normal growth medium supplemented with $60\mu M$ linoleic acid for a 48 hour period prior to heating rather than a 24 hour period.

Individual results from two experiments run in duplicate are shown on the graph.

Best fit regression lines have been fitted to the linear portions of the survival curves using the straight line equation $y = a + bx$.

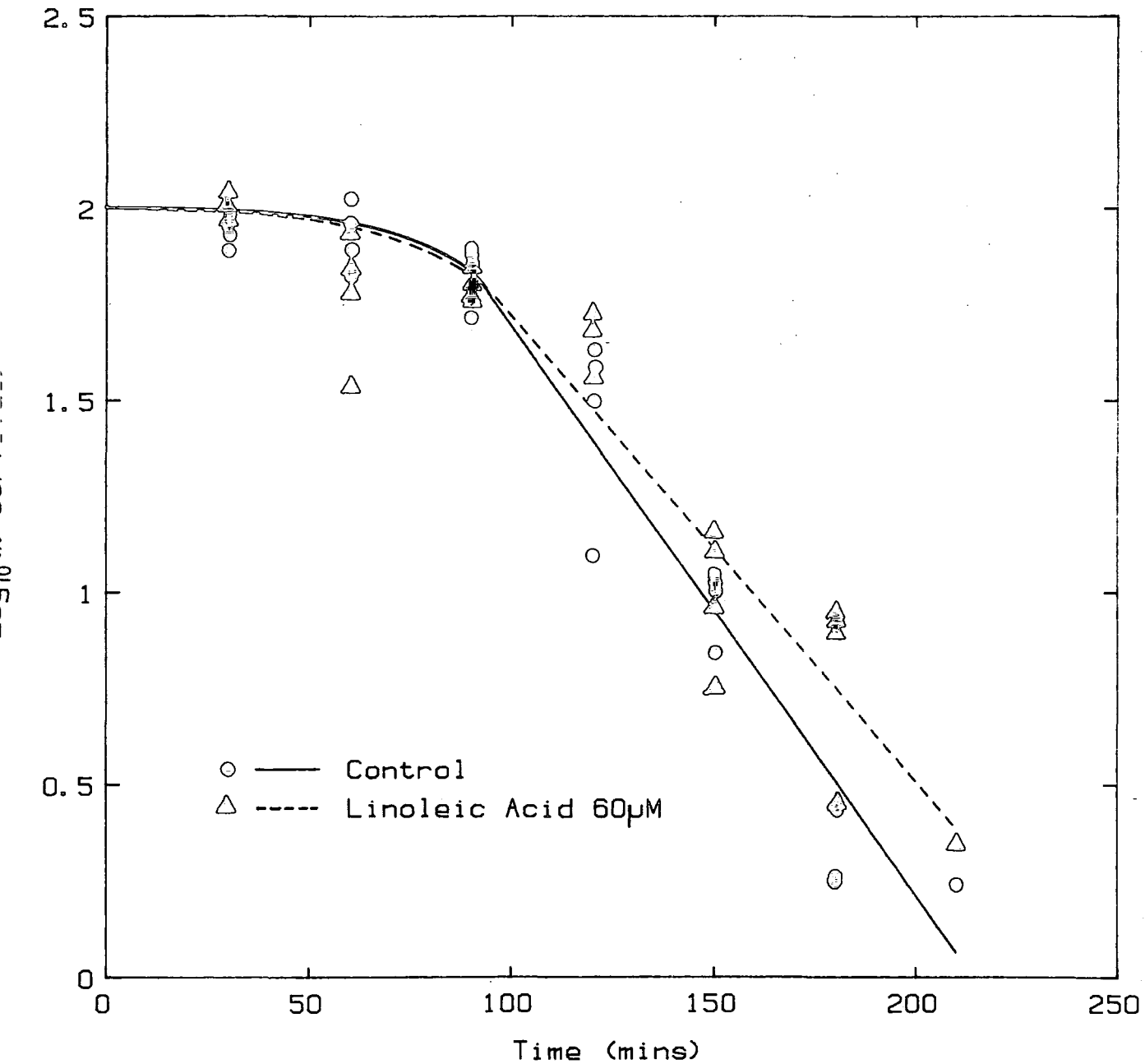

2.5
2
1.5
1
0.5
0
0
50
100
150
200
250
Time (mins)
Control
Linoleic Acid 60μM

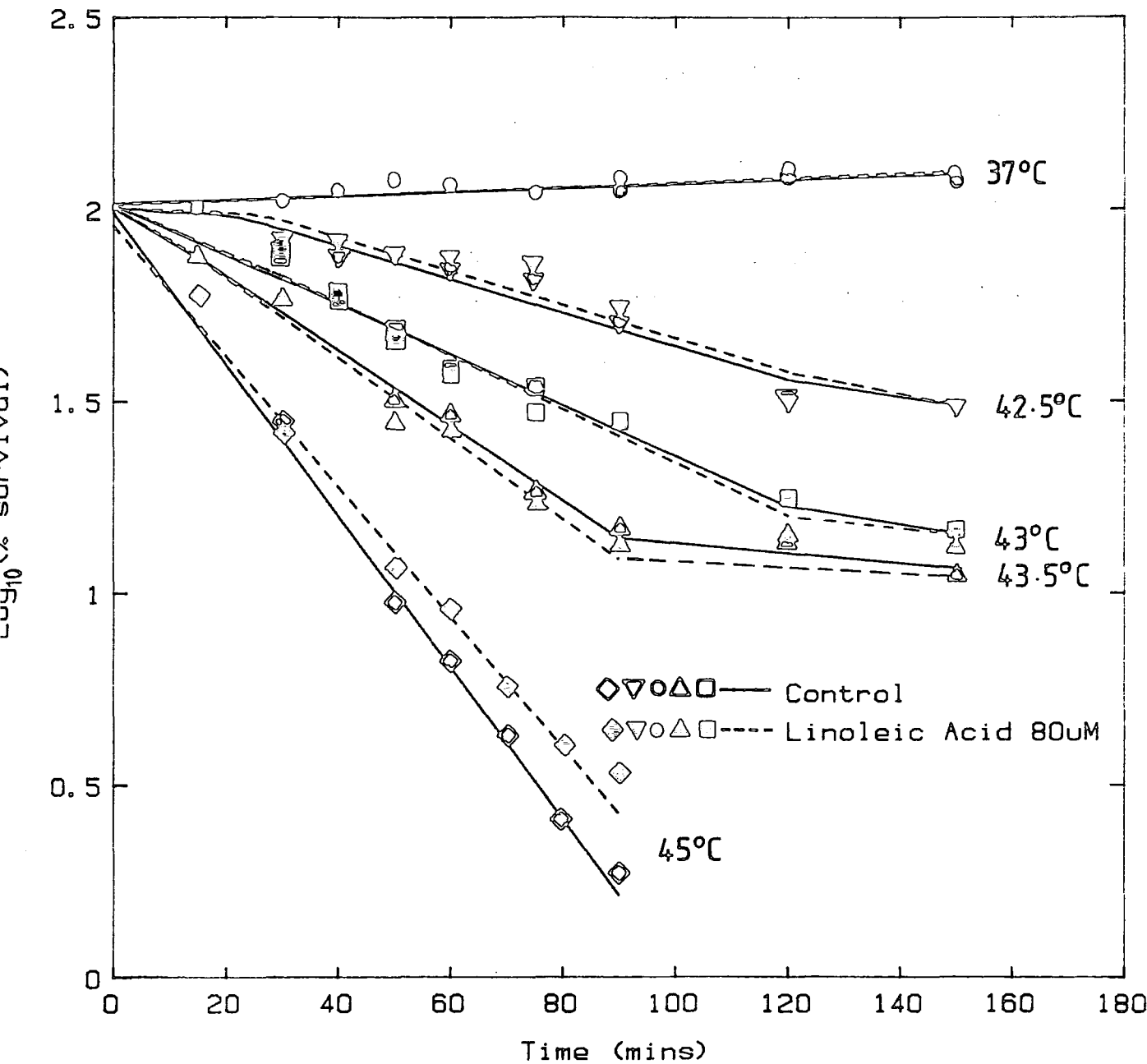
Figure 3.20

The effect of a 36 hour period of supplementation with $80\mu M$ linoleic acid on the thermal sensitivity of HTC cells exposed to a range of temperatures in 24-well plates

Experimental details are as described in the legend of figure 3.8 except that on day 1 cells were exposed to normal growth medium supplemented with $80\mu M$ linoleic acid to achieve a 36 hour period of supplementation prior to heating on day 3.

Each point on the graph represents the mean value derived from a total of 8 replicate wells run in two separate experiments.

Best fit regression lines have been fitted to the linear portions of the survival curves using the straight line equation $y = a + bx$.


2.5
2
1.5
1
0.5
0
0
20
40
60
80
100
120
140
160
180
Time (mins)
37°C
42.5°C
43°C
43.5°C
45°C
Control
Linoleic Acid 80uM

lost. Regression lines fitted to the linear portions of the survival curves (with regression correlation coefficients, $|r| > 0.94$) in fact suggest a decrease in thermal sensitivity since LD_{90} values for control and supplemented cells are 146 and 159 minutes respectively. There is also a change in D_0 values from approximately 68 minutes for control cells to 83 minutes with supplemented cells.

Since the only experimental conditions that gave rise to a significant increase in the thermal sensitivity of HTC cells in terms of LD_{90} values in flasks were based on a 36 hour period of supplementation with $60\mu M$ linoleic acid prior to heating, it was decided to conduct a further series of hyperthermic experiments in plates over a range of temperatures following a 36 hour period of supplementation with the slightly higher concentration of $80\mu M$ linoleic acid. It was hoped that the slightly higher concentration of linoleic acid, which had not been found to markedly affect cell growth in plates (figure 3.13) might lead to a more pronounced sensitisation of the cells to heat.

The results of the 36 hour supplementation hyperthermic studies conducted in plates are shown in figure 3.20. It is evident that supplementation with $80\mu M$ linoleic acid had little effect on the hyperthermic response of HTC cells as measured by the colorimetric assay. Regression lines fitted to the linear portions of survival curves (with regression correlation coefficients, $|r| > 0.90$) in fact suggest that at $42.5°C$ and $45°C$ thermal sensitivity of the cells was slightly decreased by supplementation with linoleic acid, with this effect at $45°C$ being more pronounced after longer periods of heating. The most consistent, though not significant ($p > 0.05$), increase in thermal sensitivity produced by supplementation is seen at a temperature of $43.5°C$ where supplementation served to reduce the LD_{50} value by approximately 4 minutes. The significant increase in thermal sensitivity seen with the clonogenic assay at $43°C$ was not observed with the colorimetric assay at the same temperature.

3.4 Discussion

From the results it would appear that newborn bovine serum is an important component of the growth medium of HTC cells (figure 3.1) and that the level of serum in the growth medium can influence the nature of hyperthermic survival curves that are produced (figure 3.4). Fluctuations in pH that would appear to

occur at elevated temperatures and are more marked where smaller volumes of media are involved (Table 3.1) can be minimised by employing a suitable buffering system. In addition, whilst cell passage number does not appear to influence the hyperthermic survival of HTC cells (figure 3.5) the presence of fungizone in the growth medium during the heating process would appear to increase the thermal sensitivity of these cells (figure 3.3). This information was of great importance in establishing the methodology that was subsequently adopted in morphological and supplementation hyperthermic studies. It helped to ensure that the effects observed with control and supplemented cells on exposure to heat were indeed due to hyperthermia and not artefactual, i.e. due to factors other than heat.

The apparent influence of serum on the heating response of HTC cells observed in the current study (figure 3.4) has been demonstrated with a number of cell types by other workers. For example, Sertich et al. (1980) have shown that CHO cells heated in defined growth medium in the absence of 20% (v/v) foetal bovine serum (FBS) were more sensitive to hyperthermic treatment at $43°C$ than control cells maintained in medium with 20% (v/v) FBS. Similarly, Van Dongen and Van Wijk (1986) working with synchronised mouse neuroblastoma (Neuro-2A) cells found that the presence of 10% (v/v) foetal calf serum during heat treatment served to increase the thermoresistance of G_1 phase cells relative to cells grown under conditions of serum depletion.

The results of the single study with the polyene antibiotic fungizone (figure 3.3) at a concentration of $2.5\mu g/ml$ are in agreement with Hahn et al. (1977) who showed that at $43°C$ (but not at $41°C$) this antibiotic effectively killed mammalian cells *in vitro* at doses up to $10\mu g/ml$. They suggested that the greatly enhanced cell killing reflected an interaction between the drug and hyperthermia at the level of the cells' plasma membrane, since this drug is known to bind to accessible sterols (particularly cholesterol) within the membranes of sensitive cells. Mondovi et al. (1969) using the polyene antibiotic filipin also showed that at $43°C$ this agent substantially reduced oxygen consumption of Novikoff hepatoma cells over control cells not treated with the antibiotic.

Hyperthermic studies have been conducted on a wide range of cultured cells including Chinese hamster ovary (CHO) and V79 cell lines (Gerweck, 1977; Sa-

pareto et al., 1978), murine L1210 leukemia cells (Guffy et al., 1982) and various rat hepatoma cell lines (Schamhart et al., 1984). In keeping with the results obtained with the clonogenic assay in the current study, the survival response of almost all cell lines studied when plotting Log_{10} cell survival against time of exposure to the hyperthermic temperature has been shown to be characterised by a shoulder region followed by a straight line exponential portion. Different cell lines, and even cell lines originating from the same tissue, have been found to show different heat sensitivities. For example, CHO cells exposed to $43°C$ produce D_0 values in the range 11 to 26 minutes (Sapareto et al., 1978; Gerweck, 1977), whilst V79 Chinese hamster cells have been found to have much higher D_0 values in the range 60 to 80 minutes (Schulman and Hall, 1974; Johnson, 1974). In the current study D_0 values for control cells at $43°C$ were found to be in the order of 60 minutes. Schamhart et al. (1984) reported D_0 values of approximately 30 minutes for HTC cells heated at $43°C$. The lower value may be because these workers used a different source of culture medium and foetal calf serum as opposed to newborn bovine serum in their experiments, or may reflect inherent differences in different subclones of HTC cells.

The two types of hyperthermic survival curves generated by the clonogenic assay (figure 3.7) and colorimetric assay (figure 3.8) were clearly very different. For example, whilst the clonogenic assay survival curves were characterised by marked shoulders over the temperature range $43.5°C$ to $45°C$, these shoulders were very slight or absent in the colorimetric assay. At the current time the exact reasons for the existence of the shoulder are not well understood although several workers have postulated that the shoulder on heat survival curves implies an ability of the cells to sustain sublethal damage (Hahn, 1982). If this is the case then it could well be that the lower survival seen with the colorimetric assay at short exposure times to $43.5°C$-$45°C$ reflects a temporary impairment of mitochondrial function which is still evident at 3 days post heating, but which by 10 days post heating has been repaired. Similarly, an increase in temperature should reduce the width of the shoulder. Consideration of the shoulder in figure 3.7 shows this to be the case.

Survival curves produced by the colorimetric assay at $42.5°C$, $43.5°C$ and $44°C$ show a further difference to those produced by the clonogenic assay at these

temperatures. It is evident that the survival curves produced by the colorimetric assay at these temperatures are biphasic and at longer exposure times show an enhanced cell survival relative to the clonogenic assay survival curves. This enhanced survival relative to the clonogenic assay may reflect the presence of cells which are metabolically active in the colorimetric assay at 3 days after heating but which are reproductively incompetent in the clonogenic assay at 9 days after heating. However, work by Vidair and Dewey (1988) with CHO cells heated in the plateau phase of growth has suggested two distinct modes of hyperthermic cell death which may help to account for the biphasic nature of hyperthermic cell death observed with the colorimetric assay at certain temperatures. These two workers reported a 'rapid' mode of cell death which predominated during the first few days post heating and a 'slow' mode of death which became evident at a much later stage, typically 6 to 7 days post heating.

Since the colorimetric assay was performed at 3 days post heating, then clearly if all the cells were dying by a rapid mode of death it would be expected that a more or less linear increase in cell death would occur with time. If, however some cells were affected by the slow mode of death then at the 3 days post heating assay period they would still be metabolically active and hence recorded as cells that had survived the hyperthermic insult, despite the fact that they were going to die. This proportion of cells could be fairly small in the population and hence have a fairly insignificant effect at the shorter time points where a larger number of cells survive. However, at the longer time points, where fewer cells survive their contribution will become more apparent causing the deviation from linearity observed. This explanation could also account for the higher D_0 values seen with the colorimetric assay. Vidair and Dewey were also able to demonstrate that as the heat dose was increased and the surviving fraction decreased, the rapid mode of cell death predominated. This finding could explain why the survival curve at $45°C$ produced by the colorimetric assay (figure 3.8) appears to show a linear response with time, as it would suggest that the proportion of cells being lost via the slow death mode is insignificant. It would also explain the gradual decrease in the plateau seen as the temperature increases from $42.5°C$ to $44°C$.

Clearly the major reasons for the differences in the hyperthermic survival curves generated by the clonogenic assay (figure 3.7) and colorimetric assay (fig-

ure 3.8) would therefore seem to reflect differences in the parameters used to assess cell survival (reproductive activity versus metabolic activity) and, because hyperthermic cell death is thought to be characterised by two distinct modes of death, differences in the time at which the assays were performed post heating (10 days versus 3 days).

It would seem that the version of the colorimetric assay adopted in the current study does not reflect the total hyperthermic cell death that is likely to occur, since a proportion of cells (represented by the plateau regions in the survival curves of figure 3.8) which are thought to die after longer incubation periods post heating are recorded as surviving the hyperthermic insult under the present conditions. One solution to this problem might appear to be to leave the plates for longer periods of time after heat treatment before performing the assay. However, if this were done then whilst cells surviving the heating process could well remain in the exponential phase of growth, control cells would undoubtedly reach confluency in wells and enter a stationary phase of growth leading to inaccuracies in results since these are interpreted on the basis of differences in absorbance between control and treated cells assumed to be in the same phase of growth.

The surface morphology changes that were observed with control cells following heating at $43.5°C$ for various time periods up to 80 minutes (figure 3.6) are consistent with the findings of other workers. Bass et al. (1978) studied the surface morphology of Chinese hamster ovary (CHO) cells cultured as monolayers following heating at $43°C$ for 3 hours and found that the surface of these cells was characterised by a reduction in the number of microvilli, the presence of 'blebs' and decreased adherence to the substratum. Mulcahy et al. (1981) carried out a quantitative study of the distribution of such cell surface alterations in P388 ascites tumour cells following heating at $43°C$ in suspension culture for various periods of time up to 3 hours. By establishing an arbitrary set of 10 morphological patterns to describe the broad spectrum of surface alterations observed, they were able to demonstrate a quantitative shift in the morphological distribution of surface alterations. For example, following 3 hours of heating at $43°C$, 75% of cells were classified as having a very irregular shape, with a very rough pitted surface and showing signs of fragmentation.

Borrelli et al. (1986), heating synchronous G_1 CHO cells at 45.5°C for various periods of time from 3 to 20 minutes, observed varying degrees of membrane blebbing ranging from blebbed cells virtually indistinguishable from control cells in appearance to those with blebs larger than the cell itself. They also showed that the proportion of cells exhibiting blebbing and the mean diameter of the blebs increased with heat duration, and that cells with blebs larger than 50% of the cell diameter did not survive to form colonies. It is interesting to note that in the current study with HTC cells the appearance of blebs on the surface of cells after approximately 80 minutes of heating at 43.5°C (figure 3.6) coincided with the time at which a marked decrease in cell survival began to be observed for these cells (figure 3.7) suggesting the importance of the plasma membrane as a site for hyperthermic damage.

The alteration in growth rate that was observed in the present study with HTC cells in the presence of fatty acids (figures 3.9, 3.10 and 3.11) has been demonstrated by a number of other workers. Spector et al. (1979) showed that supplementation of a culture medium containing 10% (v/v) foetal bovine serum with palmitic, linolenic or arachidonic acid reduced the growth rate of human skin fibroblasts, whilst supplementation with similar concentrations of oleate or linoleate did not affect the growth rate. In mouse fibroblast LM cells (Doi et al., 1978), supplementation with saturated fatty acids of longer than 15 carbons ($100\mu M$) was found to decrease growth whereas linoleic and arachidonic acids at the same concentration had no inhibitory effect. These workers have suggested that growth correlates with the unsaturated fatty acid content of the plasma membrane, with inhibition occurring when less than 50% of the acyl groups of plasma membrane phospholpids are unsaturated fatty acids.

In trying to establish a correlation between the thermosensitivity of cells and fatty acid supplementation procedures, workers have employed a variety of parameters as indicators of thermosensitivity. For example, Hidvegi et al. (1980) modified the membrane lipid composition of P388 ascites tumour cells by feeding host animals with diets containing either unsaturated fatty acids (UFA) or saturated fatty acids (SFA). Both kinds of ascites cells were then treated *in vitro* at 37°C, 42°C or 43.5°C for 30 to 60 minutes and the cell killing effect of hyperthermia was assessed by transplantation of cells into recipient mice. The

length of survival of the mice was taken as indicative of the thermosensitivity of supplemented cells. On this basis, since a temperature of $43.5°C$ produced a much longer survival of mice receiving ascites cells derived from the UFA diet rather than from the SFA diet these workers concluded that UFA increased the thermal sensitivity of the cells. However, whilst direct modification of the ascites cell plasma membrane composition under these conditions was demonstrated in this study by isolating plasma membranes from the cells, extracting the lipids, and analysing the fatty acid composition by Gas Chromatography, there are a number of drawbacks to the methodology that was adopted. For example, producing modified cells in one mouse, isolating them from the mouse, heating them *in vitro* in the absence of serum, cooling them and then injecting them into a second mouse subjects the cells to considerable stress in addition to the effect of heat. Thus it could be that factors other than the fatty acid levels of the ascites cell plasma membrane phospholipids are responsible for the observed results. In addition, using 'mouse survival' as an index of thermosensitivity seems to be a very indirect measurement of thermosensitivity since it is dependent in turn on a wide range of other factors such as immunological response, in its evaluation.

Guffy et al. (1982) working with L1210 murine leukemia cells used values of D_0 from hyperthermic survival curves as a measure of thermal sensitivity. They recorded D_0 values at $42°C$ of 27.5 minutes for control cells, 18.7 minutes for 22 : 6 supplemented cells and 46.2 minutes for 18 : 1 supplemented cells. As a result they concluded that supplementation of the medium with 22 : 6 caused an increase in thermosensitivity whilst supplementation with 18 : 1 caused a decrease in thermosensitivity. In these studies heating conditions would seem to be preferable to those adopted by Hidvegi et al. (1980) since supplemented L1210 cells were heated in suspension in a medium identical to that in which they were supplemented except for the absence of the supplemental fatty acid and a more quantitative soft agar clonogenic assay of cellular survival was adopted. However, since analysis of fatty acids was confined to a consideration of cellular phospholipids rather than plasma membrane phospholipids, a direct relationship between the thermosensitivity of L1210 cells and their plasma membrane fatty acid composition cannot be assumed.

Konings (1985) conducted hyperthermic studies in a similar way to Guffy et al. (1982) in an attempt to establish a correlation between thermosensitivity of mouse fibroblast LM cells and fatty acid supplementation. Cells were grown as suspensions in a serum-free medium that was supplemented with arachidonic acid (20 : 4) for a 24 hour period prior to heating in an identical medium lacking the fatty acid. He considered thermosensitivity in terms of both D_q and D_0 values and concluded that LM cells that had been supplemented with arachidonic acid showed enhanced thermosensitivity since there were differences in the results between control and supplemented cells both at the level of the shoulder (D_q) as well as at the level of the slope (D_0) of the survival curves. In addition, he also isolated the plasma membranes from control and supplemented cells and found an increase in the amount of polyunsaturated fatty acyl chains in the supplemented membrane phospholipids of approximately 38%. Hence this study clearly indicated that a relationship exists between the thermosensitivity of mouse fibroblast LM cells and their plasma membrane fatty acid composition, suggesting the importance of the plasma membrane as a site for hyperthermic damage.

In the current study, where HTC cells were grown as monolayers and supplemented and heated in serum-containing medium, LD_{90} and LD_{50} values were taken as indicators of thermosensitivity since such values should reflect the effect of both the shoulder region and the slope of the curve (Bhuyan, 1979). However, the significant decrease in survival that was seen in flasks at $43°C$ following a 36 hour period of supplementation in terms of LD_{90} values, and which was taken to mean an increase in the thermal sensitivity of these HTC cells was not accompanied by a significant decrease in D_0 value. Thus whilst this result is significant in the current study, if other parameters such as D_0 had been considered this result would not have been significant. In fact, if D_0 values are taken as indicators of thermal sensitivity then the more interesting results of fatty acid supplementation in the current study are revealed in figures 3.17 and 3.19. D_0 values suggest that a 24 hour period of supplementation with linoleic acid (figure 3.17) increases the thermal sensitivity of HTC cells, since D_0 values for control and supplemented cells are 125.8 and 96.2 minutes respectively, whilst a 48 hour period of supplementation (figure 3.19) decreases thermal sensitivity since D_0 values for control and supplemented cells are 146 and 159 minutes respectively.

The major conclusion that emerges from the current studies is that supplementation of HTC cells with linoleic acid does not produce a marked effect on the thermal sensitivity of these cells. However, what effect it does produce may well be related to the period of exposure prior to heating since the results suggest an increase in sensitivity after 24 to 36 hours of supplementation but a decrease in the sensitivity of cells at 48 hours supplementation. If the plasma membrane is an important site of hyperthermic damage it seems likely that the increase in thermosensitivity of HTC cells at $43°C$, that occurred after a 36 hour period of supplementation with linoleic acid is related in some way to the composition and physical state of the lipid matrix of the plasma membrane. This idea forms the basis of the work presented in the next chapter.

Chapter IV

Purification and Characterisation of HTC Plasma Membranes

4.1 Introduction

In order to understand the possible significance of the plasma membrane and its composition on the thermal sensitivity of HTC cells suggested by the supplementation studies of Chapter 3, it was very important to be able to isolate the plasma membranes of these cells in a state of known purity so that subsequent biochemical analyses, fluidity measurements and enzyme studies could be conducted specifically on these membranes.

Although the first isolation of a subcellular organelle occurred as early as 1871 when Friedrich Miescher isolated nuclei from broken cells by a centrifugation procedure, it was not until the 1960's when the advent of the Electron Microscope had revealed the complex membrane architecture of animal cells and as scientists became more interested in understanding the various functions of the plasma membrane, that the isolate and characterise approach was applied to plasma membranes.

Because rat liver was readily available and much was already known about this tissue, it formed the basis for many of the initial studies. As a result many of the techniques adopted in plasma membrane isolations today are based on procedures developed originally for the fractionation and identification of the major organelles in rodent liver homogenates. After Neville (1960) had established a method for isolating plasma membranes from rat liver many other workers went on to develop methods for the isolation of various plasma membranes from a wide range of solid organs and tumours (see DePierre and Karnovsky review, 1973).

However, solid tumours and organs have a number of drawbacks as a starting material for plasma membrane isolation. Firstly, they are generally composed of more than one cell type (de Duve, 1964). Indeed the liver contains approximately equal numbers of two different cell types, parenchymal and reticuloendothelial

(Kupffer) cells. Secondly, contamination of membrane fractions can arise from both the vascular and nervous systems associated with the organ. Thirdly, even the plasma membrane of an individual cell in an organ appears to be heterogeneous. For example, distinct morphological differences have been shown (Bloom and Fawcett, 1962) in the apical, lateral and basal membranes of liver parenchymal cells. This means that plasma membranes that are produced from solid tumours or organs might easily be enriched in materials from a minor cell type or form a particular pole of a cell. In addition, in many tumours the nuclei are large in relation to the cytoplasm and so are easily disrupted during homogenisation forming a gel which hinders further separation of the plasma membranes (Emmelot et al., 1974).

The development of cell culture techniques, where particular cell types are grown in isolation from other cells, has provided the solution to many of the problems associated with the isolation of plasma membranes from organs and tumours. Plasma membranes have been isolated from a number of types of cultured cells including HTC cells (Tweto et al., 1976; Lopez-Saura et al., 1978; Sauvage et al., 1981), HeLa cells (Boone et al., 1969), hepatoma cells (Koizumi et al., 1976) and epidermoid carcinoma cells of the A431 cell line (Payrastre et al., 1988).

When use is made of cultured cells in plasma membrane isolations the way in which they are grown will often determine the isolation procedure that is adopted. For example, with monolayer cultures when cell production is increased by growing cells on the surface of microcarrier beads in suspension (Chapter 2) plasma membranes may either be isolated from cells attached to the beads (Gotlib, 1982) or after the cells have been removed from the beads.

Although a number of techniques have been introduced for the isolation of plasma membranes based upon the principle of adherence of whole cells to solid supports, followed by removal of internal components (Gotlib and Searls, 1980; Cohen et al., 1980), release of plasma membranes attached to beads is often problematical.

If cells are to be removed from the microcarrier beads on which they are grown, prior to plasma membrane isolation, efficient methods are required for

the harvesting of the cells from the microcarriers and for the separation of the cells from the microcarriers. Various methods can be used to remove cells from microcarriers but any method that is adopted should ensure that damage to the cells is minimised. Very often the type of harvesting procedure adopted depends on how firmly the cells are attached to the microcarrier and on the nature of the microcarrier itself. For example incubation in a hypotonic solution can be used for harvesting cells which do not have strong adhesion properties. The osmotic shock associated with the hypotonic solution causes the cells to adopt a rounded morphology and they can then be shaken from the microcarrier. Lai et al. (1980) have used hypotonic treatment to harvest CHO cells from microcarriers. Sometimes chelating agents such as EDTA are sufficient for the removal of cells from microcarriers. However the most frequent method of removing cells from microcarrier involves the use of enzymes. Trypsin is the most commonly used general protease for this purpose. It has been used, for example, to remove Vero, BS-C-1, MRC-5 and HeLa cells from Cytodex microcarriers (Billig et al., 1984). Some microcarriers have been especially designed so that cells can be removed by enzymatic digestion of the culture surface rather than enzymatic digestion of the cell surface. Cytodex 3 microcarriers produced by Pharmacia possess a surface layer of denatured collagen which is susceptible to enzymatic digestion using collagenase. Removal of cells in this way should, in theory, increase cell viability and help maintain a greater membrane integrity.

Having removed cells from microcarriers a number of methods are available for their separation which are based on differences between the cells and microcarriers in terms of size and density. Separation may be achieved simply by differential sedimentation at unit gravity (Gebb et al., 1982). However, much higher cell yields can be obtained, without significantly altering cell viability, by employing differential centrifugation or filtration methods (Billig et al., 1984). Billig et al. (1984) have reported the recovery of up to 80% of total cells present in a culture prior to harvesting by using Ficoll-Paque in a discontinuous gradient centrifugation technique. The use of filters to separate cells from microcarriers has been reported by a number of workers (Mitchell and Wray, 1979; Ewell and Carter, 1982; Billig et al., 1984). The percentage of cells recovered by filtration processes depends on the pore size of the filter and the thickness of the microcarrier layer retained by the filter. Billig et al. (1984) showed that increasing

the pore size from $53\mu m$ to $88\mu m$ increased the yield of Vero and HeLa cells separated from microcarriers from 63% to 74% without affecting the purity of the filtrate. Both Billig et al. (1984) and Ewell and Carter (1982) have shown that greatest cell recoveries occur when the bead layer is maintained as a thin even layer.

Two major steps characterise all plasma membrane isolation procedures whether cells are derived from organs, tumours or from cell culture, where they may or may not be attached to a culture surface. Firstly, the cells must be disrupted in some way and secondly the plasma membranes must be separated from the other cell organelles and membranes.

The initial cellular disruption is a crucial step in the isolation procedure since it can profoundly affect the nature of the ensuing membrane preparation. Ideally it should produce minimal damage to intracellular organelles such as nuclei, mitochondria and lysosomes since many of these have buoyant densities close to those reported for plasma membranes (Kashnig and Kasper, 1969). In addition, the plasma membrane fragments produced should be as large as possible since this makes them easier to separate from microsomes which are the major contaminant of almost all plasma membrane preparations. (Boone et al., 1969; McKeel and Jarett, 1970).

Methods of cellular disruption tend to vary in the types of forces used to break the cells and in the composition of the medium in which cells are disrupted. A number of workers have employed cell rupturing pumps to prepare plasma membrane fractions. Cells are propelled through a narrow spring-controlled orifice in this method, which strips them of their plasma membranes. Bridgen et al. (1976) have reported the isolation of plasma membrane fractions from cultured human lymphocytes using this method.

Gas bubble nucleation, in which cells are equilibrated with inert gas at very high pressures and then returned suddenly to atmospheric pressure, has been used quite successfully in the preparation of plasma membranes from tumour cells, in particular Ehrlich ascites carcinoma cells (Wallach and Kamat, 1966). However, the drawback to this method is that it tends to produce very small plasma membrane fragments which are difficult to separate from microsomes.

Sonication has been used to achieve cellular disruption in some circumstances, e.g. rat mast cells (Cooper and Stanworth, 1976; Amende and Donlon, 1985). This method was adopted in the current study, for a short period, but was not continued since it was found to be a rather damaging process, invariably damaging both the plasma membrane and cell organelles.

One of the most successful methods of cellular disruption, and the principal method adopted in this work, involves the use of mechanical forces to break cells. Mechanical disruption is commonly achieved either in motor driven Potter-Elvejham homogenisers or hand operated Dounce homogenisers. In either case cells are subjected to a shear force as they pass between the pestle and glass wall of the homogeniser. The Dounce homogeniser, being more gentle in its action, has been used by a number of workers to disrupt cultured cells. (Bosmann et al., 1968; Boone et al., 1969).

The effectiveness of any cellular disruption process employed can often be increased by paying special attention to the composition of the medium in which the cells are disrupted. Sucrose is often present in the medium since it has been found to encourage cellular disruption and prevent aggregation of particles in the homogenate in many, but not all, instances. Similarly, disruption media are frequently buffered at pH 7.4 since lower pH values have often been found to increase the resistance of cells to cellular disruption procedures, (Bell et al., 1971). Consultation of the literature reveals many variations in the composition of the medium used to disrupt cells which often reflect particular properties of the cells being isolated.

Techniques for the separation of plasma membranes from other cell organelles and membranes are based to a large extent on the various properties of the sub-cellular particles such as weight, shape, density and surface charge. For example free-flow electrophoresis has been used to prepare plasma membranes from the stomach (Chang, 1977). However, the most common separations involve centrifugation techniques. Generally even when other techniques such as electrophoresis are employed, centrifugation is a necessary preliminary step.

Centrifugation techniques in plasma membrane isolation procedures usually involve two steps, a differential centrifugation step and a density gradient step.

In the differential centrifugation step particles are separated due to differences in sedimentation coefficients which are determined by the volume, shape and density of the particle together with the viscosity and density of the medium. Providing the suspending medium is less dense than the particles, all of the components of the mixture can be separated in pellet form by using various combinations of increasing gravitational force (g force) and time.

Although some workers have based their plasma membrane isolations on differential centrifugation alone (Carroll and Sereda, 1968), sedimentation coefficients of the various cellular organelles are not usually sufficiently different to ensure a clean separation in this way, which is why density gradient separations are generally performed. The density gradient step often involves an isopycnic separation in which the particles are centrifuged through a density gradient that encompasses their own density. The gradient in density has traditionally been established by a gradient in sucrose concentrations. Both continuous and discontinuous gradients have been used though continuous gradients, which show a continuous decrease in density from bottom to top tend to give the best separation (Wallach and Kamat, 1966).

However, sucrose gradients not only exert an osmotic pressure but involve a great deal of time and care in their preparation, and normally require long centrifugation times, often in excess of 2 hours. Consequently alternative ways of producing gradients have been sought. Gradients have been produced by employing synthetic polymers such as Ficoll-400 (Wallach and Kamat, 1966) and inert particles composed of colloidal-silica such as Ludox (Rome et al., 1979) and Percoll (Belsham et al., 1980).

Percoll is proving increasingly useful in plasma membrane isolation procedures. It consists of heterogeneous particles of colloidal-silica coated with polyvinylpyrrolidone (PVP) which renders the material completely non-toxic. Unlike sucrose, it has a low osmolality which permits precise adjustment to physiological conditions and it does not penetrate membranes thereby allowing particles to band isopycnically at their true buoyant densities. However, the great advantage of Percoll is that due to the heterogeneity of particle sizes and its low viscosity, gradients can be generated spontaneously and very rapidly in

a centrifuge which means that gradient formation and sample separation can be achieved in a single operation thereby reducing the length of the isolation procedure, and hence the time that the membranes are exposed to the harsh isolation conditions.

Amende and Donlon (1985) employed a single Percoll gradient centrifugation step to produce plasma membranes from rat liver cells that showed a 4.5-fold enrichment in the plasma membrane marker enzyme, 5′nucleotidase, as compared to the 2.8-fold obtained by Ishizaka et al. (1981) using discontinuous sucrose gradients. Chakravarthy and co-workers (1985) produced a high yield of purified plasma membrane fraction from cultured murine neuroblastoma cells that showed an 11-fold enrichment in the plasma membrane marker enzyme 5′nucleotidase on a discontinuous gradient of 5%, 25% and 35% Percoll within 1 hour of cell disruption by nitrogen cavitation.

Since Percoll has been used successfully in plasma membrane preparations with other cultured cells and would appear to produce high yields of relatively pure plasma membrane by employing simple and rapid techniques, it was adopted in the current study. Loten and Redshaw-Loten (1986) have described a purification method involving Percoll for rat liver plasma membranes which provided a useful starting point to this work with HTC cells.

In order to be able to isolate a component such as the plasma membrane from a mixture there has to be some form of assay for that component. In addition, to be able to assess the purity of the final plasma membrane preparation assays must be available to measure other components present in the cell homogenate.

A wide range of 'marker' techniques have been developed that are based on the premise that each morphologically distinguishable organelle and membrane system has constituents or combinations of features that render them unique (de Duve, 1964). These techniques have made use of the morphological, biochemical, histochemical and immunological properties of the various organelles and membrane systems. For example, the presence of receptors in plasma membranes to which metabolic effectors (e.g. lectins, immunoglobulins, toxins) and polypeptide hormones bind rapidly and specifically has led to the use of radio-labelled ligands to identify plasma membranes in subcellular fractions (Chang et al., 1975).

Electron microscopy provides a qualitative assessment of contamination of subcellular fractions by nuclear envelopes since Monneron et al. (1972) have shown that these paired membranes are characterised by the attachment of amorphous material (containing DNA and chromatin) to their non-cytoplasmic surface.

However one of the most common forms of marker techniques, and one that was adopted in the current study, is that involving the measurement of enzymes that have been shown histochemically to be associated with particular membrane fractions. The advantage of this technique is that it enables a quantitative measurement to be made and is fairly easy to perform. It is important to bear in mind however that this technique does have its drawbacks such as the loss in enzyme activity and vesicular latency that can occur during cell rupture and subsequent separation of organelles (Evans, 1982). In addition, it is important to remember that the cellular location of an enzyme in one cell type is not necessarily the same as that in other cell types. For example, although 5′nucleotidase is assumed to be on the plasma membranes of mammalian cells, Wallach and Ullrey (1962) reported that this enzyme is localised exclusively in the nuclei of Ehrlich ascites carcinoma cells. Likewise, analytical fractionation studies of HTC cells (Lopez-Saura et al., 1978) have suggested that these cells do not contain typical 5′nucleotidase. Thus care must be taken in the choice of marker enzymes in plasma membrane isolation studies.

The major constituents of animal cell membranes are lipids, proteins and polysaccharides (Simon, 1974; Singer and Nicolson, 1972). Membrane functions such as enzymic, transport and receptor activities are believed largely to be mediated by proteins which can contribute 50% or more of the weight of isolated animal cell plasma membranes (Nystrom, 1973). Lipids on the other hand, are considered to be more important as structural components of membranes and are composed mainly of cholesterol and phospholipids with lesser quantities of neutral fat (Brossa et al., 1980). Plasma membrane lipids encompass up to 7 classes of phospholipid, their lyso-derivatives and neutral glycerolipids all of which exhibit varying patterns of fatty acids.

Many models have been put forward over the years to account for the structure of cell membranes. The contemporary model of membrane structure is

based on that proposed by Singer and Nicolson (1972) which pictures membrane proteins as floating in a sea of phospholipids, the latter being organised as a bilayer. From a consideration of the thermodynamics of macromolecules, properties of the proteins and lipids of functional membranes and other experimental evidence, these authors concluded that a 'fluid mosaic model' of the gross organisation and structure of the proteins and lipids of biological membranes was consistent with restrictions imposed by thermodynamics. They also suggested that the proteins in this model are a heterogeneous set of globular molecules, each arranged in an amphipathic structure, with the ionic and polar headgroups protruding from the membrane into the aqueous phase and the non-polar groups being largely buried in the hydrophobic interior of the membrane. This model has since been expanded to include cholesterol which in the normal liquid phase has a condensing effect on the fatty acid chains of phospholipid molecules (Oldfield and Chapman, 1972). A further modification suggests that some of the proteins may be 'anchored' or their movements relatively impeded by association with the cytoskeleton.

One of the most important features of the fluid mosaic model of membrane structure centres on the dynamic state of the lipids in the bilayer. This dynamic state has been found to be influenced by a number of factors including the properties of the fatty acyl chains of the phospholipid bilayer, cholesterol content and the phospholipid polar headgroup composition (Van Blitterswijk et al., 1987) and temperature.

Many different kinds of fatty acyl chains are present in biological membranes. They are of chain length C_{16} to C_{22} and most are characterised by an even number of carbon atoms. Of these, some 35% to 40% are saturated whilst the remainder are unsaturated containing between 1 and 6 double bonds. In addition, not only are plasma membranes from different cell types characterised by different and distinctive fatty acyl compositions, but each phospholipid class of a particular plasma membrane also has a characteristic spectrum of fatty acids present (White, 1973). The purpose of this complex chemical composition is not clear but it is believed to be related to the maintenance of the physical state of the lipid matrix.

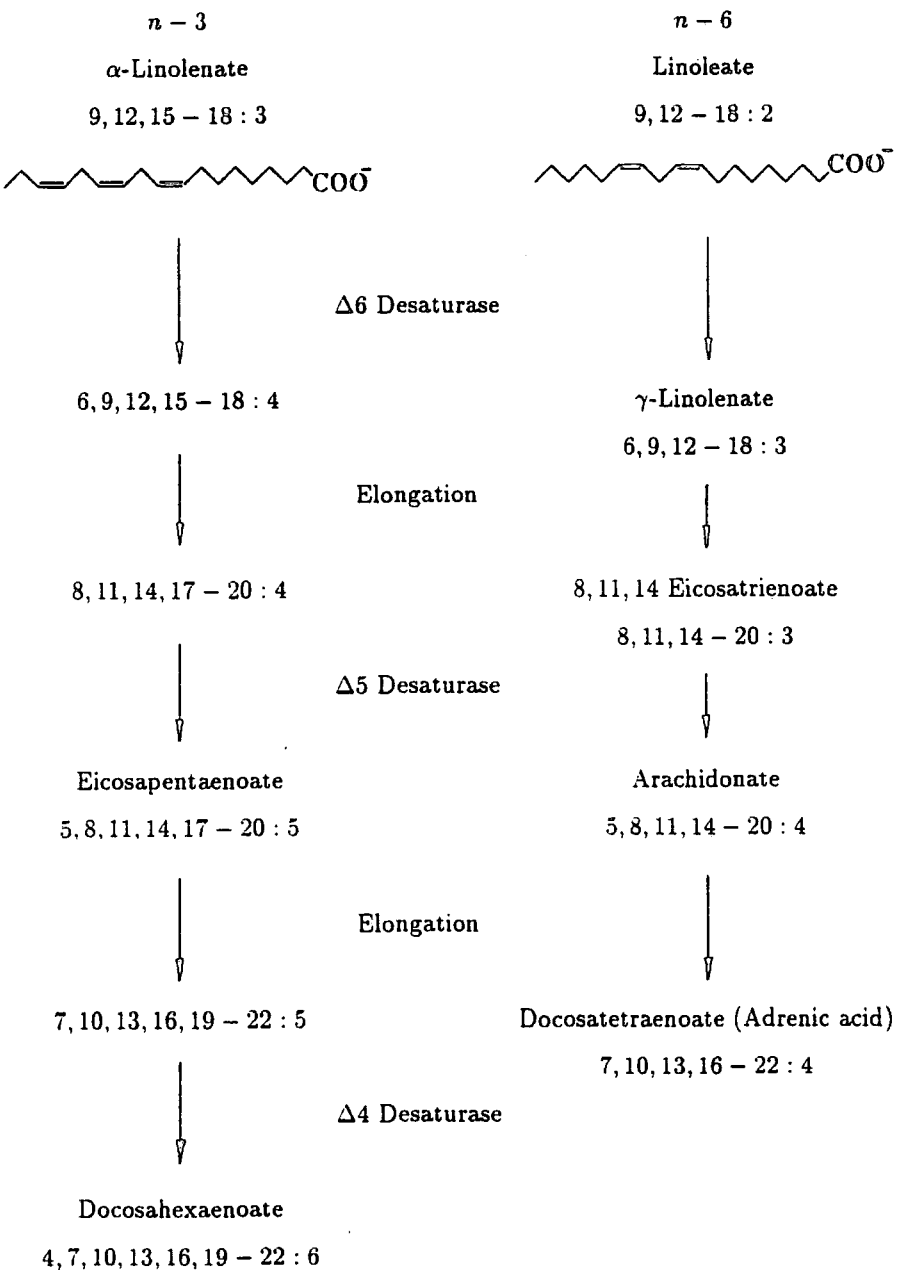
In most organisms the primary product of fatty acid biosynthesis is palmitic acid (16 : 0) and it is from this common precursor that most other fatty acids, apart from linoleic and linolenic acid (the so called essential fatty acids), found in membranes are formed by elongation and desaturation reactions. Elongation reactions involve the addition of 2 carbon units to long chain fatty acids whilst desaturation reactions involve the dehydrogenation of 2 adjacent carbons in the fatty acyl chain with a high degree of positional specificity. Eukaryotic cells have been found to contain up to 4 distinct desaturase enzymes each of which has its own positional specificity. The most common of these, $\Delta 9$ desaturase, inserts a double bond between carbons 9 and 10 (from the carboxyl end) of saturated fatty acyl-CoA derivatives. The other desaturases that exist are $\Delta 6$, $\Delta 5$ and $\Delta 4$ desaturases.

The fatty acid composition of animal cells is profoundly influenced by the availability of linoleic and α-linolenic acids. Since higher animals are unable to synthesise these two fatty acids, they are obtained from the diet in the case of animals and from the extracellular fluid in the case of cells. These two essential fatty acids serve as precursors for the two main classes of polyunsaturates found in animal cells. Dietary linoleic acid serves as the precursor for the $\omega - 6$ or $n - 6$ family of polyunsaturated fatty acids, all of which have the first double bond at carbon 6 counting from the methyl end of the molecule (figure 4.1). Similarly, α-linolenic acid is the precursor for the $\omega - 3$ or $n - 3$ family (figure 4.1). Whilst the same series of enzymes desaturates and elongates the appropriate members of both families of polyunsaturated fatty acids, the two families of fatty acids are not interconvertible. Thus 18 : 3 $n - 6$ is a different fatty acid to the 18 : 3 of the $n - 3$ class. The fatty acids can however, be modified by desaturation, chain elongation and in some cases chain shortening or retroconversion to produce other members of the same fatty acid family (Rosenthal, 1987).

The attachment of a fatty acid to a phospholipid involves the action of a fatty acyl transferase using as its substrates a lysophospholipid and fatty acyl-CoA. The fatty acyl-CoA substrate is usually formed by the action of fatty acyl-CoA synthetase on free fatty acids either synthesised de novo or derived from dietary sources. Available evidence strongly supports the existence of at least two types of acyl-CoA : lysophospholipid acyltransferase (Lands and

Figure 4.1

Major pathways for desaturation and elongation of essential fatty acids in HTC cells



The positions of double bonds with respect to the carboxyl carbon are indicated in front of the two numbers which indicate the number of carbon atoms : number of double bonds. The notation for fatty acid family $(n-3, n-6)$ is based on the location of the double bond proximal to the methyl or w carbon which is unchanged by the process of desaturation and elongation. (Redrawn from Rosenthal, 1987).

Crawford, 1976). One specifically attaches an acyl group to the 1 position of 2-acylglycerolphospholipids, while the other esterifies an acyl group to the 2 position of 1-acylglycerolphospholipids. Under optimised *in vitro* conditions, the enzyme that places an acyl group at the primary hydroxyl position appears to be selective for saturated fatty acids, while the acyltransferase activity at the 2 position seems to prefer unsaturated acids (Lands and Crawford, 1976).

Most cells also contain enzymes capable of hydrolysing fatty acids from phospholipids. Such enzymes usually display positional specificity with those designated phospholipase A_1 cleaving the fatty acid from the $sn-1$ position of glycerophospholipids and those designated phospholipase A_2 deacylating glycerophospholipids at the $sn-2$ position (Shinitsky, 1984).

Though some of the remarkably asymmetric distributions of fatty acids in membrane structural lipids undoubtedly arises during de novo synthesis of these compounds, the subsequent replacement of certain fatty acyl chains of the completed lipids with different ones through deacylation followed by reacylation reactions would also seem to be important in producing the striking differences seen in the fatty acyl chains of membrane phospholipids. In addition, there are numerous other reactions by which the fatty acid composition of a particular phospholipid can be affected. For example, there can be quantitatively significant transfers of entire diacylglycerol moieties from one phospholipid class to another by a number of different pathways. Sundler and Åkesson (1975) have shown that approximately 20% of the molecular species of phosphatidylcholine in mammalian liver is recruited from the phosphatidylethanolamine pool by sequential methylation of the latter phospholipid.

When culture medium contains an adequate supply of lipids, fatty acid synthesis (de novo) and cholesterol synthesis are suppressed and most of the cellular lipids are derived from the material that is taken up from the medium (Spector and Yorek, 1985). This finding has led to a large number of studies in which the fatty acyl composition of mammalian cells has been modified. The types of cells that have been modified in this manner include fibroblasts (Rosenthal, 1980), myogenic cells (Horwitz et al., 1978) and endothelial cells (Spector et al., 1980).

Such studies have shown that there is considerable variation in the extent to which different fatty acids can be increased in fatty acyl groups of phospholipids. For example, when bovine pulmonary artery endothelial cells were supplemented with palmitic acid (16 : 0) in medium containing 10% foetal bovine serum (FBS), the phospholipid 16 : 0 content and total saturated fatty acid content increased only slightly, whereas with oleic acid (18 : 1) the 18 : 1 content of the endothelial phospholipids increased by 60% (Kaduce et al., 1982). When 3T3 cells were supplemented with linoleic acid (18 : 2) in 10% newborn bovine serum whilst the level of linoleic acid increases two fold, a four fold increase in the arachidonic acid (20 : 4) content of the phospholipids was seen (Denning et al., 1982). This increase in arachidonic acid content does not occur when human skin fibroblasts are supplemented with linoleic acid (Spector et al., 1979).

Some of this variation has been found to reflect the cells' metabolic capacity for elongating and desaturating fatty acids. Although most cells in culture seem capable of carrying out elongation reactions, many cells have been found to lack one or more of the 4 enzymes involved in desaturation reactions. For example whilst $\Delta 9$ desaturase, which primarily converts stearic acid (18 : 0) to oleic acid (18 : 1), would appear to be operational in most cell lines and has been conclusively demonstrated in HTC rat hepatoma cells (Wiegand and Wood, 1975), the $\Delta 6$ desaturase which converts linoleic acid (18 : 2 $n-6$) to γ-linolenic acid (18 : 3) and α-linolenic acid (18 : 3 $n-3$) to parinaric acid (18 : 4) is reported to be absent in a number of different cell lines including several transformed mouse cell lines (Dunbar and Bailey, 1975).

Although a large number of fatty acid supplementation studies have been conducted, in many instances such studies have been confined to a consideration of the modifications produced in total cellular phospholipids with lipid extraction procedures being carried out on whole cells (Rosenthal, 1979; Yatvin et al., 1983; Nelson et al., 1986). Some workers, such as Yatvin et al. (1987), suggest that since cellular phospholipids are primarily present in the membrane fractions the total phospholipid content of a cell serves as an approximate measure of its membrane content. However, as Burns et al. (1983) have shown in their study of normal mouse tissues and membranes *in vivo*, whilst cellular phospholipids can be used to estimate the direction of plasma membrane alteration they need

not necessarily reflect the extent of the alteration. For example, Burns et al. (1983) showed that, whilst purified liver plasma membranes demonstrated diet-induced changes, the level of arachidonic acid in the plasma membrane was lower as compared to whole liver phospholipids and when animals were fed sunflower diets, rich in polyunsaturates, the plasma membranes phospholipids were less polyunsaturated as compared to the whole liver phospholipids. Thus, in the current study, changes in phospholipid fatty acyl composition have been studied using purified HTC cell plasma membrane fractions.

Fatty acid modification procedures have formed the basis of a large number of experiments in which fatty acyl group composition, membrane physical properties and membrane function are simultaneously compared in an effort to determine the nature of the relationship between these three properties.

A number of studies with prokaryotic and artificial membranes (Machtiger and Fox, 1973; Silbert et al., 1974) have suggested that changes in saturated fatty acid content result in marked changes in membrane 'fluidity'. Similar studies with cultured cells have suggested a relationship between fatty acyl composition and 'fluidity'. For example Konings (1985) working with mouse LM cells showed that plasma membranes that were enriched with polyunsaturated fatty acyl chains were more 'fluid' as compared to control plasma membranes. Furthermore, Burns et al. (1979) using L1210 leukemia cells have demonstrated that cells from animals fed a diet enriched with polyunsaturated fatty acids have a more fluid plasma membrane than cells grown in host animals fed on diet rich in saturated fatty acids.

A relationship between fatty acyl composition, 'fluidity' and response to temperature fluctuations has been suggested by a number of studies. It has been known for many years that exposure of microorganisms, plants and animals to low temperatures leads to the incorporation of increased proportions of unsaturated fatty acids in the storage lipids and in membrane phospholipids (Hazel, 1984). Sinensky (1974) using electron spin resonance (ESR) spectroscopy showed that the 'fluidity' and phase state of membrane from *Escherichia coli* remained constant at a variety of growth temperatures. He used the term 'homeoviscous adaptation' to stress both the homeostatic nature of the response and its

adaptive importance. The significance of homeostatic regulation relates to the direct effects of bilayer physical properties upon the functional properties of the membrane since bilayer lipids provide the effective solvent environment of the functional components, namely integral membrane-bound proteins. It would seem that the chemical composition of many, if not all, membranes is regulated to maintain the physical state or 'fluidity' of the bilayer within tolerable limits optimal for membrane function.

Membrane 'fluidity', although a term which is widely used in the study of membranes, is one that tends to lack a precise definition. Generally, it is taken to mean a combination of different types of mobility of membrane components which can include the flexibility of acyl chains, transverse diffusion of molecules from one monolayer to the other, the lateral diffusion of molecules in the plane of the membrane and phase transitions leading to lateral phase separations (Goldstein, 1984). Different workers have their own interpretations. In the present study, as suggested by Stubbs and Smith (1984) membrane fluidity has been interpreted in terms of the physical state of the fatty acyl chains of the phospholipids comprising the bilayer structure. An important point to bear in mind however, is that whilst 'fluidity' in this sense is likely to be directly affected by the degree of unsaturation of the fatty acyl chains and the fatty acyl chain length, it may also be influenced by other components of the membrane such as cholesterol and proteins since these are also known to influence the behaviour of fatty acyl chains. This implies that fatty acid unsaturation and membrane fluidity are unlikely to be related in a simple manner.

Membrane fluidity can be operationally defined and quantified using one of several biophysical techniques such as nuclear magnetic resonance (NMR) spectroscopy, electron spin resonance (ESR) spectroscopy and fluorescence polarisation spectroscopy techniques that employ various 'probes' that can be intercalated within the bilayer interior. The basic assumptions are first that the measured motional characteristics of the probe are sensitive to the dynamical motion or order of the surrounding hydrocarbon chains and second that the normal physical structure of the bilayer is not greatly disturbed by the probe. Each technique provides specific information on the type of motion which affects the spectroscopic property in question so that different techniques report on different

aspects of the fluid condition. Stubbs and Smith (1984) suggest that the various parameters that are extracted by the different techniques can be classified into one of two types. These are the rate of motion and the angular range of motion, or the degree of order, which in membranes are related to the acyl chain rotational mobility.

Steady state fluorescence polarisation spectroscopy, which appears to produce information on the angular range of motion (degree of order) of the fatty acyl chains, was selected in the current study since measurements can be performed on relatively small amounts of membrane sample, are highly reproducible and are relatively simple to perform (Lee, 1982). In steady state polarisation studies a fluorescent probe is inserted deep into the core of the membrane bilayer. Once the dye molecules have equilibrated in the lipid matrix of the membrane they are then excited by polarised light and emit fluorescence which is polarised parallel to the excitation light. The extent of movement of the molecules during the nanoseconds of the excited state determines the proportion of light which is depolarised on fluorescence. If the environment is very fluid then the excited molecules will be totally depolarised. If however, the environment is very rigid then no motion will take place during the lifetime of the excited state and the fluorescence polarisation will be equal to the maximum possible value for that molecule.

In the current study 1,6-diphenyl-1,3,5-hexatriene (DPH), a rigid, rod shaped fluorescent molecule was chosen as the probe. Whilst this molecule only fluoresces when it is in the hydrophobic core of the membrane and not when it is an aqueous environment, it is prone to photoisomerisation and so exposure to excitation light should only occur just before a polarisation measurement is to be taken (Lee, 1982).

One important point to bear in mind with the use of probe techniques such as fluorescence polarisation spectroscopy is that although lipid domains of varying order have been shown to exist within many plasma membranes (Schroeder, 1983) these techniques are not able to differentiate between different microdomains but rather produce a weighted average of the probe positions, determining overall changes in the hydrophobic core of membranes.

Once again, as with fatty acyl compositional studies, fluorescence polarisation fluidity measurements have often been conducted on whole cells rather than on isolated membranes. One possible artefact of such measurements has been suggested by Collard and De Wildt (1978) and Stubbs et al. (1980), who pointed out that with whole cells there is the possibility that the probe may be taken up into cytoplasmic lipid droplets which can appear in cells fed excess fatty acids and which were apparent in the current supplementation studies with HTC cells (see Chapter 3). For example, supplementation of lymphocytes with linoleate gave a large decrease in the fluorescence polarisation of DPH labelled intact cells which was found to be due entirely to DPH located in triacylglycerol droplets in the cytoplasm (Stubbs et al., 1980). Thus in the current study fluorescence polarisation measurements were conducted on purified plasma membrane fractions.

The importance of cholesterol in determining membrane lipid fluidity has been suggested by a number of studies. Poznansky et al. (1973) have shown that in artificial membranes cholesterol interacts with the membrane phospholipids thereby reducing their cross-sectional areas, suppresses the motion of acyl chains and decreases the fluidity of the membrane. Cholesterol would appear to exhibit a dual role with regard to membrane fluidity, since it has been shown to reduce the thermal motion of acyl chains at temperatures above the phospholipid phase transition when membranes become highly fluid whereas, below this temperature, it appears to increase acyl chain mobility by disrupting the van der Waal's forces (Stubbs, 1983).

Although studies with erythrocytes have shown that changing the cholesterol : phospholipid ratio over a wide range can markedly affect the physical properties of the erythrocyte membranes producing alterations in cell surface area, curvature and lipid fluidity (Chabanel et al., 1983) relatively few studies have investigated interactions between acyl chain modification and cholesterol content in regulating membrane properties. Where such studies have been conducted conflicting results have been obtained. King and Spector (1978) working with Ehrlich ascites tumour cells and Spector et al. (1979) working with human skin fibroblasts have shown that modifications in fatty acyl composition produced by exposure of cells to a variety of fatty acids bound to albumin are not accompanied by any changes in the membrane cholesterol to phospholipid molar

ratio. Edwards-Webb and Gurr (1988), on the other hand, working with guinea pig erythrocyte membranes have shown that when guinea pigs are fed diets containing a high proportion of linoleic acid, membranes were not only enriched in linoleic acid but also contained a higher ratio of cholesterol to phospholipid. The order parameter of the membrane as determined by ESR spectroscopy was found to be unaffected by the dietary fat. These findings have led these workers to propose that any diet-induced change in phospholipid fatty acid composition may well be counteracted by a re-distribution of cholesterol in the membrane so as to maintain a constant physical state in the membrane.

Membrane associated enzyme activity has been shown to be influenced in many instances by the 'fluidity' (order) of membrane lipids (Kimelberg, 1977). For example studies with essential fatty acid deficient rats have shown that the ouabain-sensitive Na^+/K^+ATPase is increased in the submandibular salivary glands and kidneys of rats, compared to control animals (Alam and Alam, 1986). Similarly, much evidence suggests an alteration in the structure or function of membrane proteins in hyperthermic cell death. Burdon et al. (1982, 1984) reported a dramatic loss of Na^+/K^+ATPase activity in HeLa cells after 10 minutes at $45°C$. Clearly if hyperthermia produces an increase in membrane lipid 'fluidity' then this could account for the alterations in membrane proteins observed. Inactivation of membrane proteins such as enzymes and receptors will lead to an impairment of function and a loss of response by the compartment bounded by the membrane which, in time, could lead to a loss of cellular homoeostasis and, possibly, an irreversible loss of function resulting in cell death. If exposure of cells to unsaturated supplemental fatty acids leads to changes in the fatty acid composition of the plasma membrane phospholipids which exert a fluidising effect on membrane lipids, then supplementation and heat treatment together should produce a marked increase in the thermal sensitivity of membrane proteins if membrane 'fluidity' is a key factor in determining membrane associated enzyme activity.

This current chapter has a number of aims. First to develop an isolation procedure for HTC plasma membranes that will produce membranes in high yield and with a high degree of purity. Secondly, to determine the lipid composition of isolated plasma membranes derived from control and fatty acid supplemented

HTC cells. Thirdly, to determine whether any differences that exist can be correlated with a change in plasma membrane fluidity as determined by D.P.H. fluorescence polarisation measurements. Fourthly, it aims to establish whether such changes can also be correlated with an alteration in the thermal sensitivity of plasma membrane proteins. Taken together such investigations should provide an insight into possible reasons for the enhanced thermosensitivity of HTC cells observed at $43°C$ following a 36 hour period of supplementation with linoleic acid.

4.2 Materials and Methods

4.2.1 Preparation of Purified Plasma Membranes

(a) Microcarrier Culture

An appropriate number of Techne flasks (maximum of 3 per plasma membrane (p.m.) isolation) were set up as described in Chapter 2, Materials and Methods, section 2.2.2(b) with 4×10^7 cells/$1.5g$ Cytodex 2/Techne flask. When plasma membranes from fatty acid supplemented cells were required Techne flasks were supplemented with $60\mu M$ linoleic acid prepared as described in Chapter 3, Materials and Methods, section 3.2.3, for a 36 hour period prior to p.m. isolation.

(b) Isolation of cells from microcarriers

The culture medium was discarded from the flasks. Microcarrier beads were washed three times with $10mM$ Tris-HCl, pH 7.4, containing $150mM$ $NaCl$ at $4°C$. Cells were then removed from the beads by incubating the beads, with stirring, in the same buffer containing $2mM$ EDTA at $37°C$ for 15 minutes. The omission of any enzyme in this detachment procedure ensured that damage to membranes was minimised. Cell detachment was facilitated by repeated aspiration of the suspension with a $50ml$ syringe during the last three minutes of this incubation. After the beads had settled the supernatant was filtered through nylon mesh ($88\mu m$ pore size) and retained. This cell detachment procedure was repeated twice more and the supernatants combined. A cell count was performed on the isolated cells which were then pelleted by a low speed spin at $250g$ ($r_{av} = 18cm$) in an MSE Coolspin centrifuge at $4°C$.

(c) Plasma membrane isolation Methods

(i) Method 1

The isolation of HTC plasma membranes was based on the method of Loten and Redshaw-Loten (1986) with some modification. Figure 4.2 summarises the original Loten and Redshaw-Loten purification scheme and the scheme adopted in method 1.

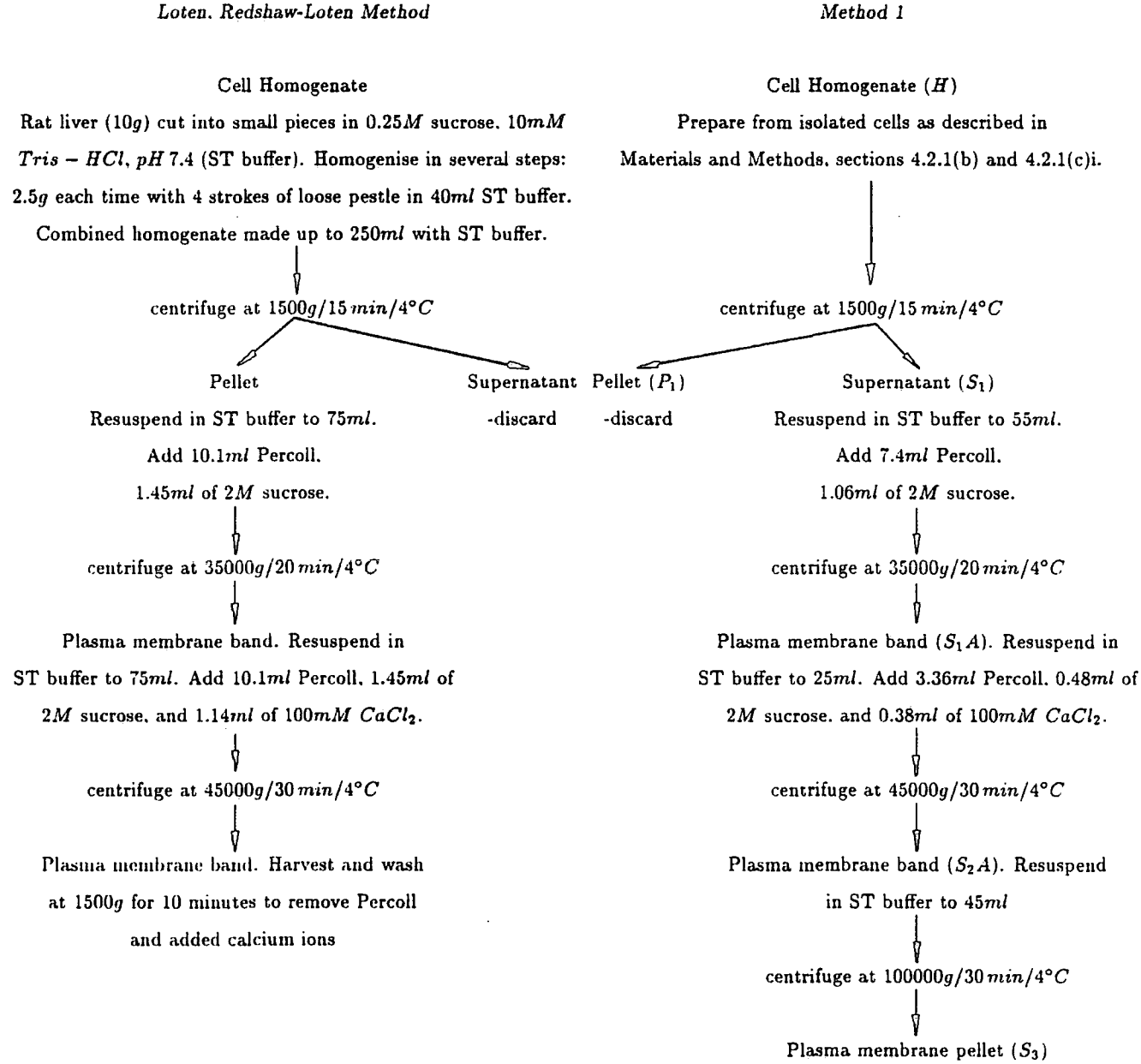
In the Loten method rat liver tissue was cut into small pieces and homogenised producing large sheets of plasma membrane nuclei etc., which were sedimented at low speeds ($1500g/15min/4°C$). The pellet was resuspended in $0.25M$ sucrose $10mM$ Tris-HCl buffer, pH 7.4 (ST buffer) and fractionated on a self forming Percoll gradient ($35\,000g/20min/4°C$) to produce a plasma membrane band. A second Percoll step was then carried out ($45\,000g/30min/4°C$) in the presence of a low concentration of calcium ions to separate DNA from the plasma membranes. Finally, the plasma membranes were harvested and washed at $1500g$ for 10 minutes to remove Percoll and added calcium ions.

In method 1, since the starting material was isolated HTC cells rather than liver tissue, the low speed spin ($1500g/15min/4°C$) of the Loten method was utilised to sediment unbroken cells since the homogenisation procedure produced small fragments of plasma membranes from the isolated cells. Consequently, the supernatant, rather than the pellet from the low speed spin was subsequently purified. In addition, after the second Percoll spin the plasma membrane was collected, diluted with ST buffer and spun down at $100\,000g$ ($r_{av} = 7.7cm$) to produce a membrane pellet.

Once a cell pellet had been obtained, as described in section 4.2.1(b), all subsequent operations were performed at $4°C$. The cell pellet containing approximately 8×10^8 cells was resuspended in $10ml$ of ice cold $10mM$ Tris-HCl buffer, pH 7.4, and left to swell for 20 minutes. This hypotonic medium which was not employed in the original Loten and Redshaw-Loten purification scheme encouraged disruption of the cells by osmotic forces thereby reducing the amount of mechanical force that subsequently had to be applied. The suspension was then homogenised in an all glass Dounce homogeniser using 20 up and down strokes

Figure 4.2

Purification schemes for rat liver and HTC cell plasma membranes



of a tight fitting (A) pestle. To return the medium to isotonicity, a final sucrose concentration of $250mM$ was produced by adding $\frac{1}{7}th$ volume of $2M$ sucrose. Following this, the suspension was rehomogenised with 20 up and down strokes of the tight fitting (A) pestle of a Dounce homogeniser and diluted to $25ml$ with $0.25M$ sucrose, $10mM$ Tris-HCl pH 7.4 (ST buffer).

The homogenate was spun at $1500g$ ($r_{av} = 19cm$) for 15 minutes in an MSE Coolspin. The pellet (P_1) was discarded and the supernatant (S_1) was diluted to $55ml$ with ST buffer. To this was added $7.4ml$ Percoll and $1.06ml$ of $2M$ sucrose. After mixing, the suspension was centrifuged at $35\,000g$ ($r_{av} = 12.3cm$) for 20 minutes in a $6 \times 38ml$ swing out rotor in a Prepspin 50 at $4°C$. The plasma membranes banded on the self-forming Percoll gradient as a clearly visible layer near the top of each tube. These layers (S_1A bands) were collected and diluted to $25ml$ with ST buffer and homogenised with 5 gentle up and down strokes of a loose-fitting (B) pestle of a Dounce homogeniser. Then $3.36ml$ Percoll and $0.48ml$ of $2M$ sucrose was added together with $CaCl_2$ ($1.3mM$ final concentration) to aid the separation of the plasma membrane from the nuclear material (Loten and Redshaw-Loten, 1986).

The suspension was mixed and centrifuged at $45\,000g$ ($r_{av} = 12.3cm$) in the $6 \times 38ml$ rotor of the Prepspin 50 for 30 minutes at $4°C$.

The resulting p.m. band (S_2A band) was collected and diluted to $45ml$ in ST buffer and centrifuged at $100\,000g$ ($r_{av} = 7.7cm$) for 30 minutes in an $8 \times 50ml$ angle rotor of the Prepspin 50 to produce a pellet (S_3). This pellet was resuspended in $3ml$ of ST buffer and stored on ice for enzymic assays or under nitrogen at $-20°C$ for any other assay procedures.

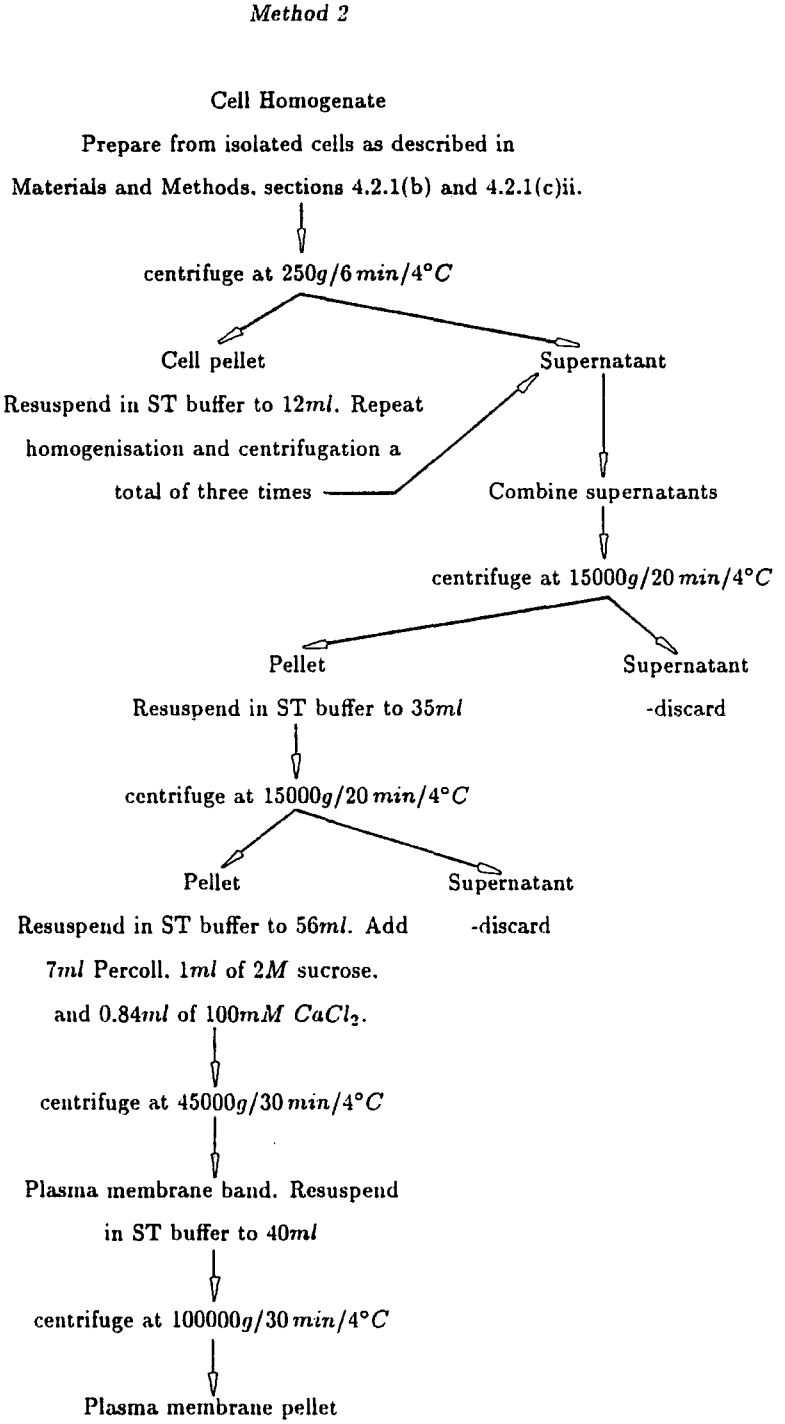
(ii) Method 2

Figure 4.3 summarises the purification scheme used in method 2.

The second method that was used to isolate HTC plasma membranes was also based on the method of Loten and Redshaw-Loten (1986) but with more extensive modifications. (Manning et al., 1989). The speed of the second spin was greatly increased to sediment the plasma membranes. Also the pellet that was obtained from the second spin was washed in ST buffer to produce a purer

Figure 4.3

Alternative Purification scheme for HTC cell plasma membranes



starting material. In addition, only one Percoll gradient was run in the presence of $CaCl_2$, to remove nuclear material. Once again the membrane bands were collected from the Percoll spin, diluted and spun down at $100\,000g$ ($r_{av} = 7.7cm$) to produce the final membrane pellet.

The cell pellet containing approximately 8×10^8 cells that was produced as described in section 4.2.1(b), was resuspended and then homogenised in $12ml$ of ice-cold ST buffer using 30 up and down strokes of a tight fitting (A) pestle of an all glass Dounce homogeniser. This operation and all subsequent operations were performed at $4°C$. After centrifugation at $250g$ for 6 minutes in an MSE Coolspin the supernatant was retained and the cell pellet was homogenised and centrifuged twice more. This 3-step approach to homogenisation was adopted to reduce the amount of damage caused to intracellular organelles.

The supernatants were pooled and centrifuged at $15\,000g$ ($r_{av} = 8.26cm$) for 20 minutes in the $8 \times 50ml$ angle rotor of an MSE Europa 24M centrifuge. The supernatant was discarded and the pellet was then 'washed' by gently resuspending it in $35ml$ of ST buffer using 3 to 4 up and down stokes of the loose-fitting (B) pestle of a Dounce homogeniser, then repeating the spin at $15\,000g$.

The pellet obtained was gently resuspended in ST buffer and then made up to $56ml$ with this buffer. After adding $7ml$ of Percoll, $0.84ml$ of $100mM$ $CaCl_2$ and $1ml$ of $2M$ sucrose, the mixture was centrifuged at $45\,000g$ ($r_{av} = 12.3cm$) for 30 minutes in a $6 \times 38ml$ swing-out rotor of a MSE Prepspin 50 centrifuge. The discrete band of plasma membranes near the top of the gradient was collected, diluted to $40ml$ with ST buffer and then recovered by centrifugation at $100\,000g$ ($r_{av} = 7.7cm$) for 30 minutes in a $8 \times 50ml$ angle rotor of the Prepspin 50 centrifuge. Finally, the pellet produced was resuspended in $3ml$ of ST buffer and stored either on ice, or at $-20°C$ under nitrogen, depending on the assays to be performed.

(d) Characterisation of Isolated Fractions

Preliminary time course studies and enzyme (protein) concentration studies were performed on all enzymes listed below that were used to characterise isolated fractions, to ensure assays of enzyme activity were made within the linear range.

(i) Na^+/K^+ ATPase assay (E.C.3.6.1.3)

Na^+/K^+ stimulated ATPase activity was determined by the method of Atkinson et al. (1973) and was measured as that activity sensitive to the presence of ouabain. Incubations were run at $37°C$ in a final volume of $1ml$. Test tubes containing $0.25ml$ of $12mM$ ATP-Tris, pH 7.2, and $0.5ml$ of $20mM$ potassium chloride, $6mM$ magnesium chloride, $200mM$ sodium chloride in $40mM$ imidazole buffer, pH 7.2, with or without $2mM$ ouabain, were preincubated for 10 minutes at $37°C$. The reaction was started by the addition of $0.25ml$ of membrane suspension. Exactly 15 minutes later the reaction was stopped by the addition of $2ml$ of acid molybdate/lubrol reagent which was prepared by mixing equal volumes of 1% (w/v) ammonium molybdate in $0.9M$ sulphuric acid with 1% (w/v) lubrol. This reagent, which is known to give a linear response with released inorganic phosphate in the range 0 to 1.2μmoles, P_i, formed a yellow soluble complex of phosphomolybdic acid and lubrol which was read at $390nm$ after precisely 10 minutes at room temperature.

The linearity of the Na^+/K^+ATPase and Mg^{2+}ATPase enzyme activities with time were investigated using freshly prepared samples of cell homogenates and final plasma membrane fractions. The assays were run as described above, in the presence or absence of $1mM$ ouabain (final concentration) but using a range of incubation periods from 5 to 45 minutes.

(ii) Alkaline Phosphodiesterase I (E.C.3.1.4.1)

The assay for alkaline phosphodiesterase I activity was based on the method of Beaufay et al. (1974). The reaction media contained a final concentration of $100mM$ $MgCl_2$, $4mM$ zinc acetate, $1mM$ p-nitrophenyl-thymidine 5′phosphate and $50mM$ glycine buffer, pH 10.2. The reaction was started, after 10 minutes preincubation at $37°C$ by the addition of $0.15ml$ of membrane suspension, pretreated with an equal volume of 0.2% (w/v) Triton X-100 for 10 minutes, to give a final assay volume of $0.75ml$. The reaction was stopped, after 30 minutes at $37°C$, by the addition of $0.75ml$ of $0.2M$ $NaOH$ and the tubes were placed on ice. The tubes were then spun at $900g$ for 15 minutes at $4°C$. The supernatant was carefully pipetted into a cuvette and the absorbance measured at $400nm$, in a dual beam Pye-Unicam SP8-100 spectrophotometer, using water as a blank.

The concentration of the p-nitrophenol produced was determined using its molar extinction coefficient of 18 300 at $400nm$.

(iii) Adenylate cyclase (E.C.4.6.1.1)

Adenylate cyclase activity of membrane suspensions were determined in a final volume of $50\mu l$. The reaction mixture used for this determination consisted of $25mM$ Tris (pH adjusted to 7.4 at room temperature with HCl), $6mM$ $MgCl_2$, $2mM$ ethyleneglycol-bis-(β-amino-ethyl ether)N, N'-tetra-acetic acid (EGTA), $0.5mM$ 3-isobutyl-1-methylxanthine (IBMX), $1mM$ Adenosine 5′ triphosphate (ATP), $1mg/ml$ bovine serum albumin (BSA) and an ATP regenerating system composed of $7mM$ phosphocreatine and $30\,units/ml$ creatine kinase. After 5 minutes preincubation at $37°C$, the reaction was started by addition of $10\mu l$ membrane suspension and the mixture incubated for a further 8 minutes at $37°C$. The reaction was terminated by the addition of $50\mu l$ of buffer, consisting of $50mM$ Tris-HCl and $12mM$ EDTA pH 7.4, and heating the reaction tube in a boiling water bath for 15 seconds, before dropping it into liquid nitrogen. The frozen reaction mixture could be stored at $-20°C$ before determination of the cyclic AMP content.

Measurement of cyclic AMP content

The cyclic AMP generated by the adenylate cyclase or added in standards and recovery experiments was determined by a procedure first described by Gilman (1970) and later modified by Tovey (1974), based on the competition between ^3H-labelled cyclic AMP and non-radioactive cyclic AMP for binding to a high-affinity cyclic AMP-binding protein.

The final composition of the assay was $0.015\mu Ci\,^3H$-cyclic AMP, $37.5mM$ Tris-HCl pH 7.0 at $25°C$, $3mM$ EDTA, $20\mu g/ml$ 3′5′-cyclic AMP dependent protein kinase and 0.025% (w/v) BSA in a final volume of $200\mu l$.

The reaction mixtures containing cyclic AMP were thawed, centrifuged in a MSE Micro Centaur centrifuge (minifuge) at 13 400g ($r_{av} = 6cm$) for 2 minutes and an aliquot of the supernatant was taken and mixed with the ^3H-cyclic AMP, Tris-HCl and EDTA. The protein kinase reconstituted in BSA was added to initiate the binding of the cyclic AMP. The tube contents were mixed on a

vortex mixer for 5 seconds and left in an ice bath for 2 to 5 hours at which time equilibrium binding had been achieved. After this period, a $100\mu l$ aliquot of the charcoal reagent composed of 2.6% (w/v) charcoal suspended in $50mM$ Tris-HCl and $4mM$ EDTA, pH 7.0 at $25°C$, was added with mixing to adsorb unbound cyclic nucleotide. The mixture was centrifuged in the minifuge for 2 minutes at $13\,400g$ ($r_{av} = 6cm$) to sediment the charcoal and a portion ($200\mu l$) of the supernatant was taken for liquid scintillation counting to determine radioactivity complexed with the binding protein. The amount of $[^3H]$-cyclic AMP complexed with the binding protein was an inverse function of the amount of unlabelled cyclic AMP present in the assay. It was necessary to quantitate this relationship by reference to a set of calibration standards.

Standardisation of cyclic AMP binding

The standard binding curve was prepared by substituting known amounts of cyclic AMP for the supernatant in the binding assay. Final standard concentrations were 0, 20, 40, 80 and $160nM$ cyclic AMP. These were not incubated for 8 minutes at $37°C$; otherwise they were treated in exactly the same way as for the incubations involving membrane suspensions.

Blanks

To determine the cyclic AMP present in the membrane suspension itself, sample blanks were carried out. These were prepared and treated as for the normal adenylate cyclase assay but in a reaction mixture which lacked ATP. The final concentration of all other constituents remained unaltered.

Recovery of cyclic AMP

Experiments designed to measure the recovery of cyclic AMP were routinely performed in reaction mixtures lacking ATP but incorporating $40nM$ cyclic AMP. The final concentration of the other constituents remained unchanged and the mixtures were incubated with membrane preparation and treated as normal.

Calculation and expression of results

The following ratio was determined:

$$\frac{counts\ obtained\ without\ unlabelled\ cyclic\ AMP(C_0)}{counts\ obtained\ with\ unlabelled\ cyclic AMP(C_x)}$$

This ratio was plotted versus the cyclic AMP present in the standards ($x = 0, 1, 2, 4, 8\ pmole$) to obtain a standard graph so that cyclic AMP in test samples could be obtained, by interpolation, using the $\frac{C_0}{C_x}$ values of the unknowns.

(iv) Cholesterol

Lipids were extracted from homogenate and final purified membrane samples by the rapid method of Bligh and Dyer (1959) described in section 4.2.2(a) below.

Unesterified cholesterol was assayed enzymatically using a BDH kit (BDH choloxidate number 1) based on cholesterol oxidase. To determine unesterified cholesterol the cholesterol ester hydrolase was omitted from the reconstituted buffered enzyme solution. Since the assay was originally designed for use with blood serum it was modified as described by Johnson (1979).

A known volume of lipid extract together with cholesterol standards (0-$0.3\mu moles$) were placed in clean conical glass tubes and evaporated to dryness under a stream of nitrogen. The lipids were redissolved in $20\mu l$ of isopropanol and preincubated at $37°C$ for 5 minutes. Reconstituted buffered enzyme solution ($0.2ml$) was then added and the tubes incubated for 10 minutes at $37°C$. The pink colour which formed was read at $500nm$ on a Pye-Unicam SP8-100 spectrophotometer in a glass cuvette with a $5mm$ path length.

(v) Succinate dehydrogenase (E.C.1.3.99.1)

Succinate dehydrogenase was assayed according to the method of Tsai et al. (1975). Duplicate cuvettes were prepared each containing $0.6ml$ of $20mM$ sodium phosphate/1% (w/v) BSA, pH 7.0 and $0.05ml$ of 1% (w/v) cytochrome *c*. After mixing they were equilibrated to $37°C$ in a water bath. Membrane suspension ($25\mu l$) was then added to both cuvettes and the cuvettes were mixed well and read against each other, in a dual beam Pye-Unicam SP8-100 spectrophotometer, to obtain a zero value. Two and a half minutes later $0.2ml$ of $10mM\ KCN$ was added to the reference cuvette and $0.2ml$ of $10mM\ KCN$ containing $50mM$ succinate was added to the test cuvette with mixing and the

increase in absorbance at $550nm$ was measured over the next 3 to 4 minutes at $37°C$. The initial rate of cytochrome *c* reduction was determined from its molar extinction coefficient of 29 705 at $550nm$.

(vi) NADPH cytochrome c reductase (E.C.1.6.2.3)

This enzyme was assayed by the method of Tsai et al. (1975) with slight modification to ensure complete exposure of this endoplasmic reticulum marker to the assay conditions.

Membrane suspensions were preincubated with an equal volume of ST buffer containing 0.1% (w/v) Triton X-100 for 10 minutes on ice. Duplicate cuvettes were prepared each containing $0.9ml$ of $0.1M$ sodium phosphate buffer, pH 7.4, containing 1% (w/v) Triton X-100 (final concentration) and $0.1ml$ of 1% (w/v) cytochrome *c*. After mixing they were equilibrated to $37°C$ in a water bath. Preincubated membrane suspension $(50\mu l)$ was then added to both cuvettes which were mixed well and read against each other to obtain a zero value. Once a stable baseline was achieved the reaction was initiated by adding $0.1ml$ of $1mM$ NADPH to the test cuvette. Distilled water $(0.1ml)$ was added to the reference cuvette to act as a blank and the cuvettes were then read immediately against each other in a dual beam Pye-Unicam SP8-100 spectrophotometer at $550nm$. The increase in absorbance was measured over the next 3 to 4 minutes. The initial rate of cytochrome *c* reduction was determined from its molar extinction coefficient of 29 705 at $550nm$.

(vii) N-acetyl β-glucosaminidase (E.C.3.2.1.30)

This enzyme assay was based on the method of Sellinger et al. (1960). In a final volume of $1ml$ the assay contained $0.1M$ sodium citrate buffer, pH 4.0, 0.1% (w/v) Triton X-100 and $6mM$ p-nitrophenyl-2-acetamido 2 deoxy-β-D-glucopyranoside. After preincubation for 5 minutes at $37°C$, the reaction was started by addition of $0.1ml$ of membrane suspension which had been pretreated with an equal volume of 0.2% (w/v) Triton X-100 on ice for 10 minutes. The reaction was stopped after 30 minutes at $37°C$ by the addition of $0.1ml$ 10% (w/v) trichloroacetic acid and the tubes were mixed well and placed on ice. The tubes were then spun at in a MSE Micro Centaur centrifuge at 13 400g ($r_{av} = 6cm$)

for 10 minutes. Following centrifugation, $0.3ml$ of $0.5M$ $NaOH$ and $0.5ml$ of $0.25M$ glycine buffer, pH 10.0, were added to $0.8ml$ of the supernatant in order to shift the pH of the solution so that the colour of the p-nitrophenol produced could develop. The intensity of the resultant colour was determined at $400nm$ on a Pye-Unicam SP8-100 spectrophotometer using water as a blank. The concentration of p-nitrophenol was calculated using its molar extinction coefficient of 18 300 at $400nm$.

(viii) Protein determinations

Protein determinations were carried out by the method of Bradford (1976) with slight modification. Sodium hydroxide ($0.1mM$ final concentration) was included in the assay procedure to solubilise membrane proteins as suggested by Simpson and Sonne (1982).

The protein reagent required was prepared by dissolving $100mg$ Coomassie Brilliant Blue G-250 in $50ml$ 95% (v/v) ethanol. After filtering, $100ml$ 85% (w/v) phosphoric acid was added. The resulting solution was diluted to a final volume of 1 litre. Freshly prepared protein reagent was routinely diluted with distilled water (3 : 1, v/v reagent : water) to ensure blank reagent values did not exceed 0.300.

A small volume of membrane suspension in ST buffer was added to a plastic minifuge tube (capacity $1.7ml$) and made up to $0.1ml$ with distilled water. To this was added $0.01ml$ of $1.1M$ $NaOH$ and the contents of the tube were mixed. Diluted protein reagent ($1ml$) was then added to the tube with mixing, and left to stand for 10 minutes at room temperature. At the end of this time and after further mixing, the contents of the tube were transferred to a cuvette and the absorbance was read at $595nm$ in a dual beam Pye-Unicam SP8-100 spectrophotometer, using water as a blank.

A standard curve was constructed using bovine serum albumin in the range 0-20μg in ST buffer.

4.2.2 Lipid Compositional Studies

Various lipid compositional studies were carried out on serum, supplemented

serum and isolated plasma membrane samples from control and linoleic acid supplemented HTC cells. The supplemented serum was prepared as described in Chapter 3, Materials and Methods, section 3.2.3. Plasma membranes were derived from HTC cells grown in microcarrier culture on Cytodex 2 beads in Techne flasks by the methodology described in Chapter 2, Materials and Methods, section 2.2.2(b). The plasma membranes were isolated by method 2 outlined in section 4.2.1(c)ii following exposure of the cells either to normal growth medium or to $60\mu M$ linoleic acid supplemented growth medium for a 36 hour period.

(a) Lipid extraction

Lipid extractions were carried out on serum, supplemented serum and isolated plasma membrane samples from control and linoleic acid supplemented HTC cells by the methodology of Bligh and Dyer (1959).

All solvents contained 0.005% (w/v) butylated hydroxytoluene (BHT) to minimise oxidation of fatty acids. To 1 volume of sample (serum samples were diluted 1.5-fold) was added 3.75 volumes of chloroform/methanol (1 : 2, v/v) with thorough mixing. Subsequent additions of 1.25 volumes chloroform and 1.25 volumes distilled water were each followed by thorough mixing. To assist the separation of the two resulting phases, a five minute low speed centrifugation ($500g$) was carried out. The bottom chloroform phase was removed and used for lipid assay procedures and thin layer chromatography studies.

(b) Assay of phospholipid

Phospholipid estimations were carried out on the lipid extracts derived from plasma membranes of control and linoleic acid supplemented HTC cells by the method of Raheja et al. (1973) with slight modification.

Aliquots of lipid extract, in duplicate, were evaporated to dryness under a stream of nitrogen in thick-walled tubes. Duplicate blank and standard assays were included. Chloroform ($0.4ml$) and chromogenic reagent ($0.2ml$) were added and the contents mixed. Each tube was heated in a boiling water bath for exactly 3 minutes, then cooled and $1.5ml$ of chloroform added. The solution was vortexed and then spun at low speed in a centrifuge ($500g$) for 5 minutes at room temperature to separate the two layers. The absorbance of the blue colour of the

lower chloroform layer was measured at $716nm$. Standard curves were prepared using dipalmitoyl phosphatidylcholine dissolved in chloroform. The assay was linear in the range 0-0.3μmoles of lipid phosphorus.

Preparation of chromogenic reagent

Reagent A: Ammonium Molybdate ($8g$) was dissolved in $60ml$ of distilled water. A few drops of concentrated hydrochloric acid were added to ensure complete dissolution.

Reagent B: Redistilled mercury ($5ml$) was added to a mixture of $20ml$ concentrated hydrochloric acid and $40ml$ reagent A. The resultant solution was mixed on a magnetic stirrer for 45 minutes and filtered producing a red brown filtrate.

Reagent C: Concentrated sulphuric acid ($100ml$) was carefully added to $20ml$ reagent A at $0°C$.

The final chromogenic reagent was prepared in a fume cupboard again at $0°C$ to prevent denaturation of the reagents by boiling. Reagent C was added very carefully, with stirring, to reagent B. A dark green solution was formed (if dark blue the reagent had been denatured during preparation). This dark green solution (25 volumes) was mixed with methanol (45 volumes), chloroform (5 volumes) and distilled water (20 volumes). The chromogenic reagent was stored in the dark at $4°C$ for up to 6 months before being replaced.

(c) Assay of unesterified cholesterol

Unesterified cholesterol present in the lipid extract produced from purified plasma membranes from control and linoleic acid supplemented HTC cells was assayed enzymatically using the BDH choloxidate number 1 kit, as described in section 4.2.1(d)iv.

(d) Thin layer chromatographic separations

A slurry of silica gel H (Kieselghur H) $0.5mm$ thick, was applied to clean glass plates $20cm$ square. Plates were air dried and then activated in an oven at 100-110$°C$ for at least one hour prior to use.

Lipid extracts from serum, supplemented serum and isolated plasma membrane samples produced from control and linoleic acid supplemented HTC cells were evaporated to dryness under nitrogen and dissolved in a small volume of chloroform. Aliquots ($50\mu l$) were then applied as 'streaks' to activated plates. Plates were run in chromatography tanks which had been 'flushed' with nitrogen just before use and all solvent systems contained 0.005% (w/v) BHT to minimise oxidation of fatty acids.

(i) Separation of fatty acids in serum samples

The solvent system used to separate fatty acids present in serum was light petroleum (bp 40-60°C)/diethyl ether/acetic acid (60 : 40 : 1, by volume). Oleic acid was run as a standard on the plates.

(ii) Isolation of total membrane associated phospholipids

Two solvent systems were used to isolate membrane phospholipids and their associated fatty acids. Sphingomyelin and phosphatidylcholine were run as standards on plates. The plate was first developed in chloroform. It was then removed and dried before being developed in a second solvent system containing chloroform/methanol (1 : 9, v/v).

After development and drying, the separated fatty acid and lipid standards were visualised by exposure of the appropriate portion of each plate to iodine vapour. Silica gel was scraped off the thin layer plates from areas in the sample lanes corresponding to the position of fatty acid and lipid standards, into small bijou bottles with a Tuf-Bond teflon seal in the screw cap.

(e) Gas liquid chromatography analysis

(i) Preparation of fatty acid methyl esters

Fatty acid methyl esters were prepared using the method of Morrison and Smith (1964) with slight modification. Sufficient boron trifluoride (14%, w/v) in methanol was added to the bijou bottles, containing the samples produced by thin layer chromatographic separations, to just wet the sample. The bottles were then sealed tightly and heated in an oven at 100°C for 15 minutes. After cooling, the volatile contaminants were allowed to evaporate in a fume cupboard

at room temperature and the fatty acid methyl esters were extracted by washing with hexane containing 0.005% (w/v) BHT and filtering the resulting extracts through glass-wool plugged pasteur pipettes which had been previously washed in the same solvent containing 0.005% (w/v) BHT. The filtrates were then either dried down under nitrogen to a minimum volume and injected onto the column or dried down completely under nitrogen, sealed and stored in the dark at $-20°C$ for future analysis.

(ii) Gas liquid chromatography of fatty acid methyl esters

Separation was carried out using a Shimadzu GC-9A series gas chromatograph. The glass columns used were $2m$ long with a $2mm$ internal diameter. They were packed with a cyanosilicone stationary phase, 10% Altech CS-5, on a chromasorb WAW 100-120 mesh support. Nitrogen was used as the carrier gas and the flow rate was $55ml$/minute. The two columns (one reference) were run in a temperature program, with one temperature ramp, as follows

$$\begin{gathered}\text{Initial Temperature} = 210°C \\ \text{Initial Time} = 10 \text{ minutes} \\ \text{Program Rate} = 4°C/\text{minute} \\ \text{Final Temperature} = 250°C \\ \text{Final time} = 5 \text{ minutes} \\ \text{Total run} = 25 \text{ minutes.}\end{gathered}$$

The resolved components were detected by a flame ionisation combustion system (hydrogen/air). Peaks were identified by comparison of their retention times with those of authentic fatty acid methyl ester standards.

The chromatogram was connected to a Trilab 2 integrator with graphics, supplied by Trivector Scientific Ltd. which was used to provide quantitative determination of the fatty acid methyl esters present in samples. The integrator calculated the area of individual peaks and the percentage contribution of each peak to the total peak area. Since peak area is directly proportional to the mass of the resolved component, percentage area contributions were an indication of the percentage mass contribution of individual fatty acids to total fatty acid mass.

4.2.3 DPH Steady State Fluorescence Polarisation Studies

(i) Membrane preparation and introduction of probe

Plasma membranes isolated from control and linoleic acid supplemented HTC cells by method 2 outlined in section 4.2.1(c)ii were resuspended in $10mM$ sodium phosphate buffer, pH 7.6. An appropriate volume of membrane suspension was added to $2.4ml$ of the same phosphate buffer in a quartz $10mm$ fluorescence cuvette to give an absorbance in the region of 0.1 at $500nm$ on a dual beam Cecil spectrophotometer. To insert the DPH probe into the membrane, $2\mu l$ of a $2mM$ solution of DPH in glass distilled tetrahydrofuran was added to the cuvette with rapid mixing. The cuvette was then incubated at $37°C$ for 15 minutes to enable the DPH to equilibrate within the membrane. Cuvettes were then cooled to $4°C$ before the start of a temperature run between 4-45$°C$. It was possible to take a number of cuvettes through each run. Consequently, control and supplemented membranes isolated from HTC cells on the same days were analysed for fluorescence polarisation under identical conditions. When cuvettes were read, they were placed in a thermostatically controlled chamber where temperatures were maintained within $\pm 0.1°C$ of the required temperature by means of a Julabo thermostated circulator. The temperature within cuvettes was measured with a calibrated thermistor. To prevent any condensation effects in the humid atmosphere at sub-ambient temperatures the thermostated cuvette chamber incorporated a facility for gassing cuvette surfaces with dry nitrogen gas.

(ii) Polarisation measurements

An analogue T-format fluorimeter (Applied Photophysic Ltd.) was used to measure steady state polarisation of DPH fluorescence. The fluorimeter was mounted on a $\frac{1}{4}inch$ aluminium optical bench. The excitation wavelength was $360nm$. The excitation path was filtered with a Corning 7-54 broadband pass filter and the emission path with a Corning 3-73 sharp out filter.

Polarisation of fluorescence (p) was calculated from the following equation:

$$p = \frac{I_{VV} - I_{VH}}{I_{VV} + I_{VH}}$$

where I_{VV} = excitation polariser vertical and emission polariser vertical.

I_{VH} = excitation polariser vertical and emission polariser horizontal.

4.2.4 Thermal Inactivation of Plasma Membrane Enzymes

HTC cells were grown by microcarrier culture on Cytodex 2 beads in Techne flasks by the methodology described in Chapter 2, section 2.2.2(b). Plasma membranes were isolated by method 2 outlined in section 4.2.1(c)ii, following exposure of the cells either to normal growth medium or to $60\mu M$ linoleic acid supplemented growth medium for a 36 hour period.

Two types of enzymatic study employing different membrane associated enzymes were conducted on the normal and linoleic acid supplemented membranes that were isolated.

(a) Thermal inactivation of Na^+/K^+ATPase

Purified plasma membranes were diluted with $0.25M$ sucrose, $10mM$ Tris-HCl, pH 7.4 (ST buffer) and $0.6ml$ of the membrane suspension was added to thermally equilibrated plastic test tubes held in a 'Forbes Bar'. This apparatus maintained a temperature gradient between 37-57°C. At each temperature there were two tubes containing either membranes from control cells or membranes from linoleic acid supplemented cells. The tubes were incubated for exactly 10 minutes and were then placed on ice to prevent further inactivation. Residual Na^+/K^+ATPase activity was assayed as described in section 4.2.1(d)i.

(b) Isothermal inactivation of Alkaline Phosphodiesterase I

Isothermal inactivation studies of the membrane associated alkaline phosphodiesterase I (Alk. PDE I) enzyme were run at 64°C (± 0.1°C) in glass tubes. An appropriate volume of membrane suspension was equilibrated in a water bath set at 37°C (± 0.1°C). This membrane suspension was then diluted in a glass test tube in a specified volume of ST buffer, which had been equilibrated at

a temperature in excess of the final test temperature, such that on mixing the temperature of the suspension registered $64°C$ immediately when placed in a water bath set at $64°C$. The glass tube containing the membrane suspension was capped to avoid evaporatory loss during the period of heating. Samples ($200\mu l$) were withdrawn from test tubes containing control or linoleic acid supplemented membranes set up in this way, at various time intervals over a 55 minute period, and placed on ice to prevent further inactivation.

Once the final sample had been obtained, $200\mu l$ of ST buffer containing 0.2% (w/v) Triton X-100 was added to each sample on ice. The test tubes were left for a further 10 minutes on ice and then residual Alk. PDE I activity was assayed as described in section 4.2.1(d)ii.

4.3 Results

4.3.1 Plasma Membrane Isolation Procedures

When the method of Gotlib (1982) for the isolation of cell plasma membranes on microcarrier culture beads was attempted in the current study very low yields of fairly impure HTC plasma membranes were obtained. In consequence, HTC cells were always removed from the microcarrier beads on which they had been growing prior to plasma membrane isolation.

(a) Plasma membrane enrichment

Two enzyme activities generally accepted as plasma membrane markers are the Na^+/K^+ATPase and the 5′nucleotidase (Amende and Donlon, 1985). However, whilst Tweto et al. (1976) reported the presence of 5′nucleotidase in HTC cells, its presence was not detected in the current study nor has it been detected in HTC cells by Lopez-Saura et al. (1978). Consequently, Na^+/K^+ATPase was used as the plasma membrane marker in initial studies. At a later stage, alkaline phosphodiesterase I and cholesterol were also assayed as plasma membrane components (Lopez-Saura et al., 1978). In addition, a determination of forskolin-activated adenylate cyclase was made since this enzyme appears to be located in the plasma membrane of many cell types including rat hepatomas (Emmelot and Bos, 1971).

The yield of plasma membrane was determined by the recoveries of the marker enzymes, whilst purification was based on enrichment of specific activities of these enzymes relative to the total cell homogenate. In the following text, reference will be made to the fractions obtained with method 1 in terms of the notations given in figure 4.2.

Figures 4.4(a) and (b) show the time course studies produced for Mg^{2+} ATPase and Na^+/K^+ATPase with HTC cell homogenates and purified plasma membrane S_3 fractions respectively, which were produced by method 1. It can be seen in both cases that whilst the Na^+/K^+ATPase enzyme showed a linear response with time, the Mg^{2+}ATPase enzyme did not. Some unidentified factor limits the activity of the Mg^{2+}ATPase enzyme within approximately 5 minutes of incubation at $37°C$. Emmelot et al. (1966) suggested that Mg^{2+}ATPase does not give a linear response with time due to the phenomenon of 'product inhibition', that is caused by its reaction product ADP. Clearly to obtain a true estimate of Mg^{2+}ATPase activity this enzyme should have been assayed after a period of 2 to 3 minutes of incubation at $37°C$, alternatively, use could have been made of an ATP-regenerating system to remove the ADP product. Since Na^+/K^+ATPase is the more reliable plasma membrane marker (Evans, 1982), as Mg^{2+}ATPase has been found in a number of locations other than the plasma membrane, conditions were optimised for the measurement of the former enzyme. Consequently, Na^+/K^+ATPase activity was measured after a 15 minute incubation period once a reasonable level of activity had been established.

(i) Method 1

Table 4.1 shows the specific activity, purification and percentage recovery of the Na^+/K^+ATPase from the various fractions described in figure 4.2, produced by method 1. It can be seen that based on this marker, plasma membranes would appear to be obtained in fairly high yield (approximately 37%) and were purified approximately 13-fold over the original homogenate.

Examination of the other fractions reveals that virtually 90% of the activity of the homogenate is obtained in the S_1 fraction after the first low speed spin. Whilst the S_1A fraction is purified approximately 3-fold over the S_1 fraction there is a substantial 46% loss in activity during the first Percoll spin. The subsequent

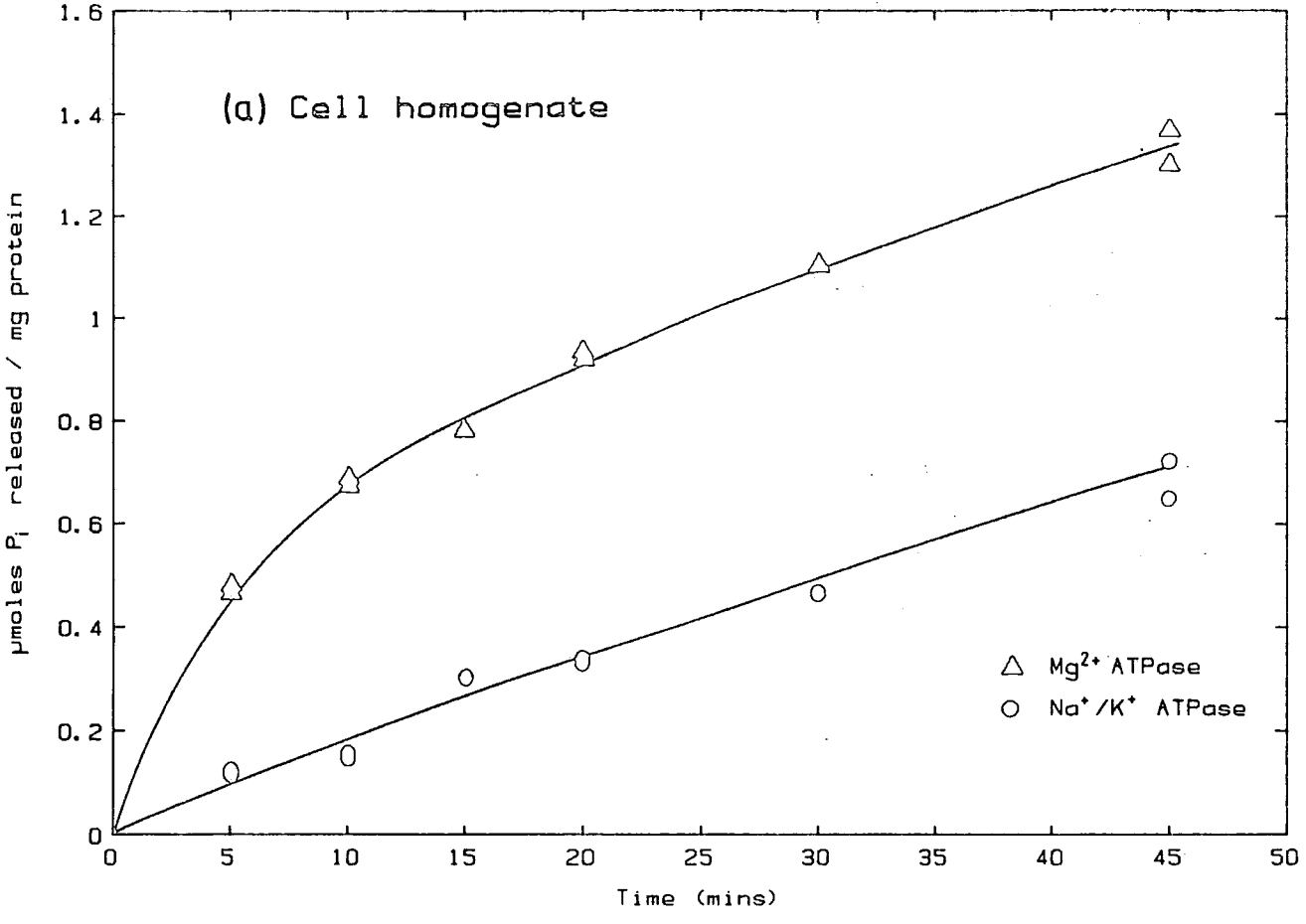
Figure 4.4

Mg^{2+}ATPase and Na^+/K^+ATPase activity in HTC cell homogenates and purified plasma membranes

HTC cell homogenates and purified plasma membranes fractions were prepared by method 1 outlined in Materials and Methods, section 4.2.1(c)i. Figure 4.4 shows the effect of varying the incubation period on the activity of the Mg^{2+}ATPase and Na^+/K^+ATPase enzymes in the cell homogenate fraction (figure 4.4(a)) and purified plasma membrane fraction (figure 4.4(b)). Enzyme assays were conducted as described in Materials and Methods, section 4.2.1(d)i.

In figure 4.4(a) individual results of a single experiment are shown.

In figure 4.4(b) each point represents the mean value $\pm$ *S.E.M.* for three separate membrane preparations.


(a) Cell homogenate
µmoles P_i released / mg protein
Time (mins)
Mg^{2+} ATPase
Na^+/K^+ ATPase

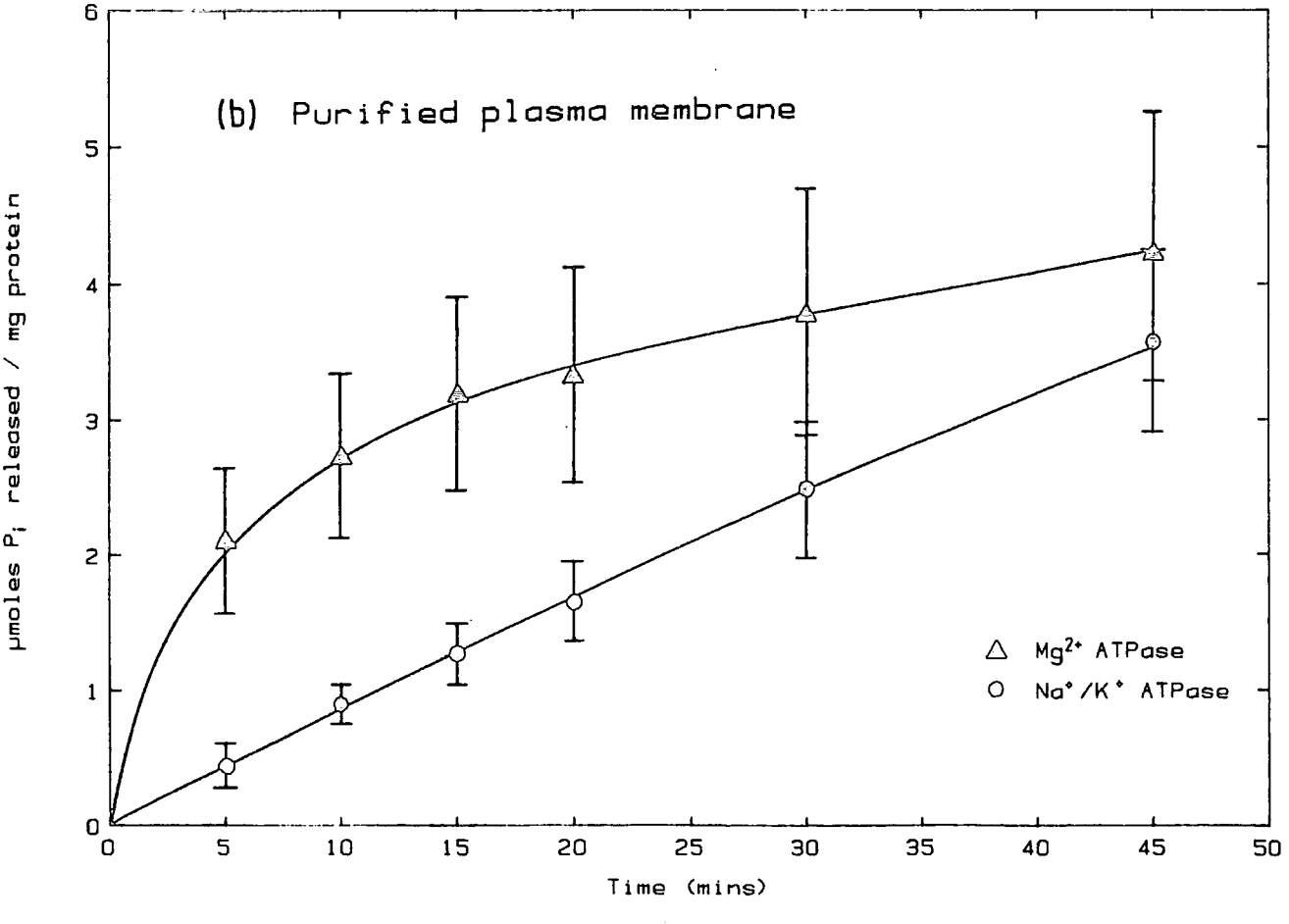

(b) Purified plasma membrane
µmoles P_i released / mg protein
Time (mins)
Mg^{2+} ATPase
Na^+/K^+ ATPase

Table 4.1

Na^+/K^+ATPase activity of isolated fractions produced from HTC cells by method 1

Table 4.1 shows the purification of plasma membranes from HTC cells produced by method 1 described in Materials and Methods, section 4.2.1(c)i. The purification is indicated by the enrichment of the marker enzyme Na^+/K^+ATPase, which was assayed as described in Materials and Methods, section 4.2.1(d)i. The fractions $H - S_3$ correspond to the notation given in figure 4.2.

Fraction	Number of Preparations	Specific Activity μmoles P_i liberated $/mg$ protein/min	Purification (fold)	Recovery (%)
H	5	0.032 ± 0.007	1.0	100
P_1	1	0.034	0.785	4.1
S_1	2	0.039	0.945	89.15
S_{1A}	2	0.123	3.05	43.25
S_{2A}	4	0.257 ± 0.056	7.21 ± 0.828	46.33 ± 14.77
S_3	4	0.380 ± 0.135	12.5 ± 1.806	36.48 ± 15.25

Results represent mean value $\pm$ $S.E.M.$

S_2A and S_3 fractions produced from the second Percoll spin and final high speed spin respectively lead to further increases in enzyme purity with only slight loss in the activity recovered.

(ii) Method 2

Because only 49% of the activity measured in the S_1 fraction was recovered in the S_1A fraction after the first Percoll spin, improvements were sought to method 1. In the original Loten and Redshaw-Loten (1986) method, on which this isolation procedure was based, a low speed pellet obtained from a rat liver homogenate was fractionated on the first self forming Percoll gradient. Although a smaller yield of plasma membranes was obtained (approximately 20%) in their method as judged by recovery of the three marker enzymes, 5′nucleotidase, alkaline phosphodiesterase I and adenylate cyclase, than was the case in method 1, the purification figure was much higher at approximately 27-fold.

In the current study where the interest lay in conducting further biochemical analysis specifically on plasma membrane material, purification of the isolated plasma membranes was a more important consideration than yield. By employing a similar homgenisation procedure to the original Loten and Loten-Redshaw method which was different to that of method 1, by increasing the speed of the second spin above that of the corresponding initial speed employed by Loten and Loten-Redshaw and method 1, by washing the pellet obtained from the second spin in ST buffer, and by employing one Percoll gradient rather than two as in method 1, the plasma membrane isolation procedure of method 2 served to increase the purity of the final membrane fractions produced.

Table 4.2 shows the specific activities, purification and percentage recoveries of up to four putative plasma membrane markers studied in the initial cell homogenates and final membrane fractions produced by method 2 for both normal and linoleic acid supplemented HTC cells.

Normal membranes and linoleic acid supplemented membranes show similar patterns of purification as judged by the Na^+/K^+ATPase and alkaline phosphodiesterase I enzymes. In the case of the Na^+/K^+ATPase enzyme the specific

Table 4.2

Various plasma membrane activities of isolated fractions produced from control and linoleic acid supplemented HTC cells by method 2

Tables 4.2(a) and (b) show the purification of plasma membranes from control cells and linoleic acid supplemented HTC cells respectively produced by method 2 described in Materials and Methods, section 4.2.1(c)ii. Supplemented cells were exposed to $60\mu M$ linoleic acid for 36 hours prior to plasma membrane isolation. The purification is indicated by the enrichment of the following marker enzymes: Na^+/K^+ATPase, alkaline phoshodiesterase I and adenylate cyclase. Purification was also monitored by cholesterol. Cholesterol and enzyme assays were conducted as described in Materials and Methods, section 4.2.1(d).

(a) Control membranes

Membrane marker	Number of Preparations	Specific Activity* Homogenate	Specific Activity* Final Pellet	Purification (fold)	Recovery (%)
Na^+/K^+ATPase	8	0.037 ± 0.003	0.552 ± 0.058	15.09 ± 1.46	28.55 ± 3.72
Alkaline Phosphodiesterase I	8	0.044 ± 0.005	0.251 ± 0.032	5.69 ± 0.244	10.38 ± 0.666
Cholesterol	5	0.029 ± 0.005	0.186 ± 0.026	6.35 ± 0.617	12.62 ± 1.24
Adenylate Cyclase	1	1.14×10^{-6}	1.344×10^{-5}	11.79	22.5

* Specific activity for Na^+/K^+ATPase is in μmoles P_i liberated/mg protein/min and for cholesterol in μmoles/mg protein. Specific activity for adenylate cyclase is in μmoles cAMP produced/mg protein/min. Specific activity for Alkaline Phosphodiesterase I is in μmoles p-nitrophenol produced/mg protein/min.

(b) Linoleic acid supplemented membranes

Membrane marker	Number of Preparations	Specific Activity μmoles P_i liberated* /mg protein/min		Purification (fold)	Recovery (%)
		Homogenate	Final Pellet		
Na^+/K^+ATPase	3	0.033 ± 0.002	0.490 ± 0.028	14.86 ± 1.18	32.48 ± 3.31
Alkaline Phosphodiesterase I	3	0.035 ± 0.009	0.290 ± 0.001	6.04 ± 0.159	12.81 ± 0.874

Results in both tables represent mean value $\pm$ *S.E.M.*

* Refers to Na^+/K^+ ATPase

Specific activity for Alkaline Phosphodiesterase I is in umoles p-nitrophenol produced /mg protein /min.

activities of the normal and supplemented membranes were 0.552 and 0.490 respectively, with purification factors of 15.09 and 14.86. These purification figures are approximately 1.2-fold higher than those obtained in method 1, although the recovery of the enzyme is approximately 6% less. The specific activities and purification figures for the alkaline phosphodiesterase I enzyme, although comparable in the final pellets of both the normal and supplemented membranes, were much lower than the corresponding Na^+/K^+ATPase figures. The specific activities of alkaline phosphodiesterase I in normal and supplemented membranes were only 0.251 and 0.290 respectively, with purification factors of 5.69 and 6.04. In addition, there was a relatively low recovery of this enzyme of approximately 11% as compared to the approximate 30% recovery obtained with Na^+/K^+ATPase. It is also evident that whilst the normal membranes show a slightly, though not significantly higher purification than the supplemented membranes with respect to the Na^+/K^+ATPase the reverse is true with the alkaline phosphodiesterase I enzyme.

Results obtained with cholesterol as the putative plasma membrane marker in normal membranes support the alkaline phosphodiesterase I results since cholesterol measurements suggest a 6.35-fold purification of the plasma membrane relative to total homogenate. On the other hand, the single forskolin-stimulated adenylate cyclase activity recorded for the normal plasma membrane final pellet is more in agreement with the Na^+/K^+ATPase results since the activity was purified approximately 12-fold with a recovery of 23% in this membrane fraction as compared to the 15-fold purification and 29% recovery seen with the Na^+/K^+ATPase enzyme. It is unfortunate that only one assay of adenylate cyclase was achieved in the current study. This was because assays for putative plasma membrane markers other than Na^+/K^+ATPase and alkaline phosphodiesterase I were not developed until a late stage in the study with the result that time did not permit more than one assay to be carried out. It is because this result would appear to support the Na^+/K^+ATPase study that it has been included for comparison.

(b) Contamination with other membranes

Table 4.3 shows the specific activities, purifications and percentage recover-

Table 4.3

Microsomal, mitochondrial and lysosomal contamination of HTC cell plasma membranes produced by method 2

Table 4.3 shows the extent of plasma membrane contamination with mitochondria, lysosomes and endoplasmic reticulum in HTC cell plasma membranes produced by method 2, described in Materials and Methods, section 4.2.1(c)ii. The level of contamination from these three sources is indicated by the enrichment of the marker enzymes succinate dehydrogenase, N-acetyl β-glucosaminidase and NADPH-cytochrome *c* reductase respectively, which were assayed as described in Materials and Methods, section 4.2.1(d).

Membrane marker	Number of Preparations	Specific Activity*		Purification (fold)	Recovery (%)
		Homogenate	Final pellet		
Succinate dehydrogenase	2	0.0122	0.0044	0.394	0.63
N-acetyl β-glucosaminidase	2	0.365	0.358	0.97	1.57
NADPH-cytochrome *c* reductase	2	0.0195	0.0335	1.76	2.81

Results represent mean values

* Specific activity of N-acetyl β-glucosaminidase is in μmoles p-nitrophenol produced/*mg* protein/*min*. Succinate dehydrogenase and NADPH-cytochrome *c* reductase specific activities are in μmoles cytochrome *c* reduced/*mg* protein/*min*.

ies of the marker enzymes succinate dehydrogenase, N-acetyl β-glucosaminidase and NADPH-cytochrome *c* reductase used to monitor contamination of the final membrane with mitochondria, lysosomes and endoplasmic reticulum respectively. Once again, it is unfortunate that time did not permit more than two assays to be carried out on membrane material prepared by method 2. In addition, it is appreciated that assays for other contaminating membranes such as the nuclear envelope and peroxisomal material should ideally have been conducted but such studies were not feasible in the time scale available. However, it can be seen that plasma membrane contamination from the three sources that were investigated was very low. It is evident that endoplasmic reticulum was the most significant contaminant of the plasma membrane preparation, since it was purified marginally (1.76-fold) by the procedure and showed the highest recovery (2.8%) of all contaminating membranes. Mitochondrial contamination was the least significant of the three contaminants assayed showing a 0.4-fold purification and a 0.7% recovery, whilst the lysosomal contaminant showed a slightly higher purification (1-fold) and a 1.6% recovery.

4.3.2 Lipid Compositional Studies

Table 4.4 reveals the free fatty acid composition of newborn bovine serum, derived from several batches of supplied serum, and the composition of linoleic acid supplemented medium prepared as described in Chapter 3. It can be seen that there was a marked increase in the level of linoleic acid present in the supplemented serum from approximately 6% to 60% of total free fatty acid, indicating that the supplementation procedure was enriching the level of linoleic acid in serum as intended.

Having established that the supplementation procedure was producing a significant increase in the level of linoleic acid present in serum, the next step was to establish whether supplementation of HTC cells was also producing a change in the fatty acid composition of the plasma membrane phospholipids. Table 4.5 shows the relative proportions of the major fatty acids in plasma membrane total phospholipid fractions derived from control and linoleic acid supplemented HTC cells. It can be seen that plasma membrane phospholipids from control cells are characterised by high levels of oleic acid (18 : 1) and palmitic acid (16 : 0),

Table 4.4

Free fatty acid composition of serum and linoleic acid supplemented serum

Linoleic acid supplemented newborn bovine serum (NBS) was prepared as described in Chapter 3, Materials and Methods, section 3.2.3. The free fatty acid composition of NBS and linoleic acid supplemented NBS was determined as described in Materials and Methods, section 4.2.2.

Fatty Acid	Relative % weight	
	Newborn Bovine Serum (NBS)	Linoleic Acid supplemented-NBS
16 : 0	27.0 ± 1.3	16.8 ± 5.2
16 : 1	1.7 ± 1.7	trace
18 : 0	22.8 ± 2.2	9.7 ± 2.6
18 : 1	32.6 ± 2.6	11.0 ± 3.5
18 : 2	6.0 ± 1.5	62.2 ± 9.7
18 : 3	1.3 ± 0.1	n.d.
20 : 0	3.4 ± 1.7	trace
20 : 1	3.3 ± 1.7	trace
20 : 4	trace	n.d.
22 : 0	0.2 ± 0.2	n.d.
22 : 1	2.8 ± 1.4	n.d.

Number of replicates = 4

Results represent mean value ± $S.E.M.$

trace = detectable level but less than 0.1%

n.d. = none detected

Table 4.5

Fatty acid composition of plasma membrane phospholipids from control and linoleic acid supplemented HTC cells

Plasma membranes were purified by method 2 outlined in Materials and Methods, section 4.2.1(c)ii from control cells and from supplemented cells that had been exposed to $60\mu M$ linoleic acid for a 36 hour period. The fatty acid composition of the plasma membrane phospholipids from control and supplemented cells was determined as described in Materials and Methods, section 4.2.2.

Fatty Acid	Relative % weight	
	Control plasma membranes	Linoleic Acid supplemented plasma membranes
16 : 0	27.7 ± 3.5	29.4 ± 2.3
16 : 1	9.2 ± 5.5	6.2 ± 2.6
18 : 0	20.1 ± 3.5	21.5 ± 3.7
18 : 1	40.0 ± 3.2	25.4 ± 5.0
18 : 2	2.2 ± 0.9	16.1 ± 1.5
18 : 3	trace	0.4 ± 0.1
20 : 0	0.3 ± 0.3	0.4 ± 0.2
20 : 1	n.d.	trace
20 : 4	0.3 ± 0.3	0.3 ± 0.3
22 : 0	n.d.	0.2 ± 0.2
22 : 1	n.d.	0.1 ± 0.1

Number of replicates = 4

Results represent mean value ± *S.E.M.*

trace = detectable level but less than 0.1%

n.d. = none detected

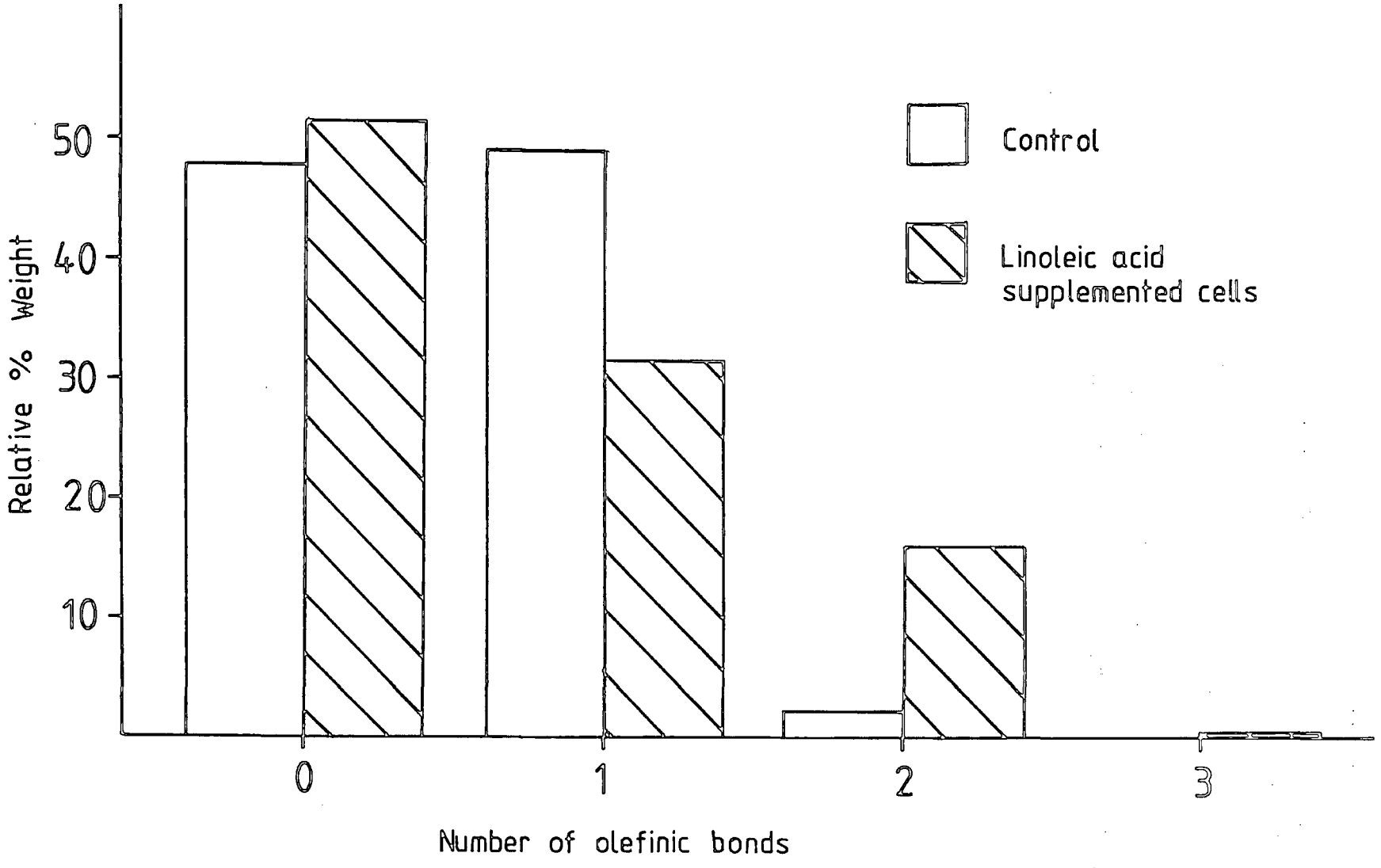
and by low levels of arachidonic acid and the two essential fatty acids; linoleic (18 : 2) and linolenic (18 : 3) acids. In addition, it is evident that the fatty acid composition of the plasma membrane phospholipids of HTC cells supplemented with 10% (v/v) newborn bovine serum (NBS) is generally similar to the free fatty acid composition of the NBS used in the preparation of the culture medium (compare Tables 4.4 and 4.5). Whilst the proportions of palmitoleic (16 : 1) and oleic (18 : 1) acids were increased in the cell plasma membrane and the proportion of linoleic acid (18 : 2) was decreased compared to that present in the free fatty acids of NBS, these differences were not statistically significant ($p > 0.05$). The proportion of arachidonic acid in the cell plasma membrane was similar to that in the medium. These results suggest that HTC cells are capable of absorbing the free fatty acid in the medium and incorporating them into membrane phospholipids, without substantial alteration of the fatty acid profile. Statistical analysis of the data in Table 4.5 by Student's t-test following arc-sine transformation indicates that supplementation of HTC cells with $60\mu M$ linoleic acid for a 36 hour period produced a significant decrease ($p < 0.05$) in the level of oleic (18 : 1) acid in the plasma membrane phospholipids from 40% to 25% and a significant increase ($p < 0.05$) in the level of linoleic acid from 2% to 16%. The level of arachidonic acid, a metabolic product of linoleic acid, and other fatty acids did not show any significant change as a result of supplementation.

It was also of interest to determine whether supplementation of HTC cells with $60\mu M$ linoleic acid for a 36 hour period produced a change in the proportions of saturated and unsaturated fatty acids present in the plasma membrane. Figure 4.5 shows the degree of unsaturation versus saturation of the plasma membrane phospholipid fatty acyl chains. It can be seen that the proportion of saturated fatty acids in the control and supplemented membranes was very similar, remaining at approximately 50%. The major difference is in the type of unsaturation with control membranes containing greater levels of mono-unsaturated fatty acids than the supplemented membranes which were characterised by higher levels of di- and tri-unsaturated fatty acids. Statistical analysis of the data by Student's t-test following arc-sine transformation reveals that the level of saturated fatty acids in control membranes is not significantly different ($p > 0.05$) to the level in supplemented membranes.

Figure 4.5

Fatty acid unsaturation of HTC cell plasma membrane phospholipids

The figure shows the proportions of olefinic (unsaturated) bonds present in the fatty acids of plasma membrane phospholipids derived from control and linoleic acid supplemented HTC cells that were treated as described in the legend of table 4.5. This figure has been constructed from the data shown in table 4.5.


Relative % Weight
50
40
30
20
10
0
1
2
3
Number of olefinic bonds
Control
Linoleic acid
supplemented cells

Since fatty acid chain length is a factor that can influence plasma membrane physical properties it was another important aspect to consider. Figure 4.6 shows the carbon chain length of plasma membrane phospholipid fatty acyl chains. It is evident that the chain length of plasma membrane phospholipids is not altered as a result of supplementation of HTC cells. Analysis of the data by Student's t-test following arc-sine transformation reveals that the proportion of both C-16 and C-18 chain lengths in control versus supplemented membranes is not significantly different ($p > 0.05$).

The fact that some fatty acid supplementation studies have reported alterations in plasma membrane cholesterol : phospholipid ratios whilst others have not, highlighted the importance of determining this parameter in the current study. Table 4.6 shows the cholesterol/phospholipid molar ratios obtained for plasma membranes from control and linoleic acid supplemented HTC cells. It is evident that there was no significant difference in the cholesterol/phospholipid molar ratios obtained since the value for control membranes was 0.455 ± 0.021 whilst the corresponding value for supplemented membranes was 0.406 ± 0.093.

Figure 4.7 shows the steady state fluorescence of DPH in plasma membranes from control and linoleic acid supplemented cells over the temperature range 4-45°C. The lower polarisation values obtained with plasma membranes purified from supplemented cells together with the difference in intercept values obtained suggest that the plasma membranes derived from linoleic acid supplemented cells were less ordered (more 'fluid') than those of control cells. Supplementation of HTC cells with 60μM linoleic acid for a 36 hour period prior to plasma membrane isolation served to shift the curve by approximately 5°C along the temperature axis. The fact that there is no statistical difference in the slope of lines generated suggests that the effect of fatty acid supplementation on membrane order does not alter across the range of temperatures measured.

Figure 4.8 shows the results of thermal inactivation studies conducted on the Na^+/K^+ATPase enzyme of purified plasma membranes produced from control cells or from supplemented cells that had been grown in microcarrier culture and exposed to 60μM linoleic acid supplement for a 36 hour period prior to isolation. It is evident that this form of inactivation study which produces a sigmoidal

Figure 4.6

Fatty acid chain length of HTC cell plasma membrane phospholipids

The figure shows the proportions of fatty acid chain lengths present in plasma membrane phospholipids derived from control and linoleic acid supplemented HTC cells that were treated as described in the legend of table 4.5. This figure has been constructed from the data shown in table 4.5.

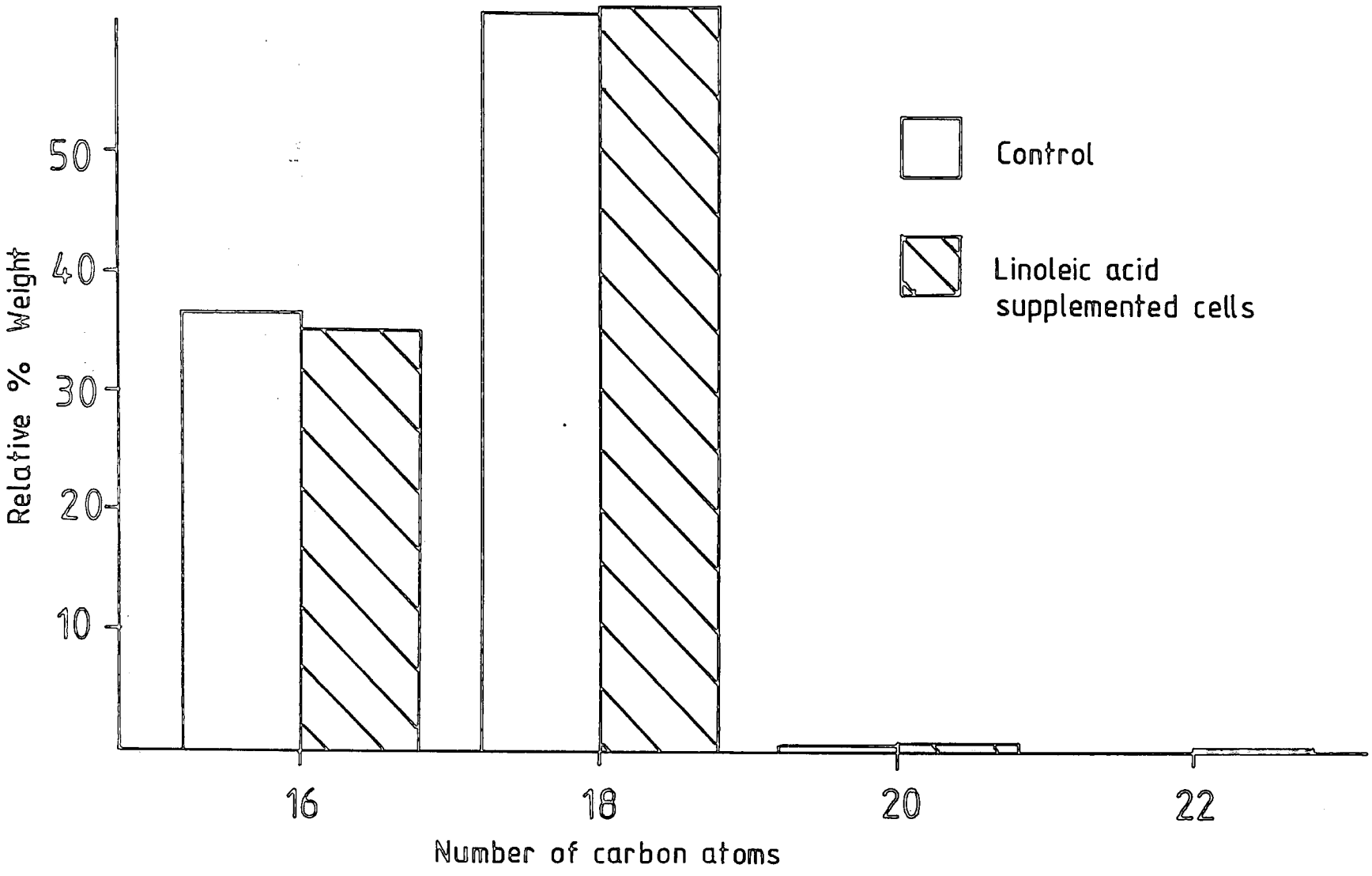

Relative % Weight
50
40
30
20
10
16
18
20
22
Number of carbon atoms
Control
Linoleic acid
supplemented cells

Table 4.6

Cholesterol/phospholipid molar ratios for control and linoleic acid supplemented HTC cell plasma membranes.

Plasma membranes were purified by method 2 outlined in Materials and Methods, section 4.2.1(c)ii from control cells and from supplemented cells that had been exposed to $60\mu M$ linoleic acid for a 36 hour period. Cholesterol, phospholipid and protein assays were conducted as described in Materials and Methods, sections 4.2.1(d) and 4.2.2.

Membrane type	Cholesterol /Protein μmoles/mg	Phospholipid /Protein μmoles lipid P/mg	Cholesterol /Phospholipid Molar ratio
Control	0.226 ± 0.029	0.497 ± 0.063	0.455 ± 0.021
Linoleic Acid -supplemented	0.201 ± 0.046	0.494 ± 0.013	0.406 ± 0.093

Number of replicates = 3

Results represent mean value $\pm$ $S.E.M.$

Figure 4.7

Steady state fluorescence polarisation of D.P.H. in plasma membranes from control and linoleic acid supplemented HTC cells

Plasma membranes were purified by method 2, outlined in Materials and Methods, section 4.2.1(c)ii from control cells and from supplemented cells that had been exposed to $60\mu M$ linoleic acid for a 36 hour period.

The figure shows the effect of temperature on the polarisation of D.P.H. in the temperature range 4-45°C. The plot is derived from two separate membrane preparations.

Best fit regression lines were fitted to the data using the straight line equation $y = a + bx$.

Values of a (intercept on y axis) and b (gradient) are given below.

Membrane type	a	$b \times 10^2$
Control	0.394	−0.409
Linoleic acid supplemented	0.371	−0.406

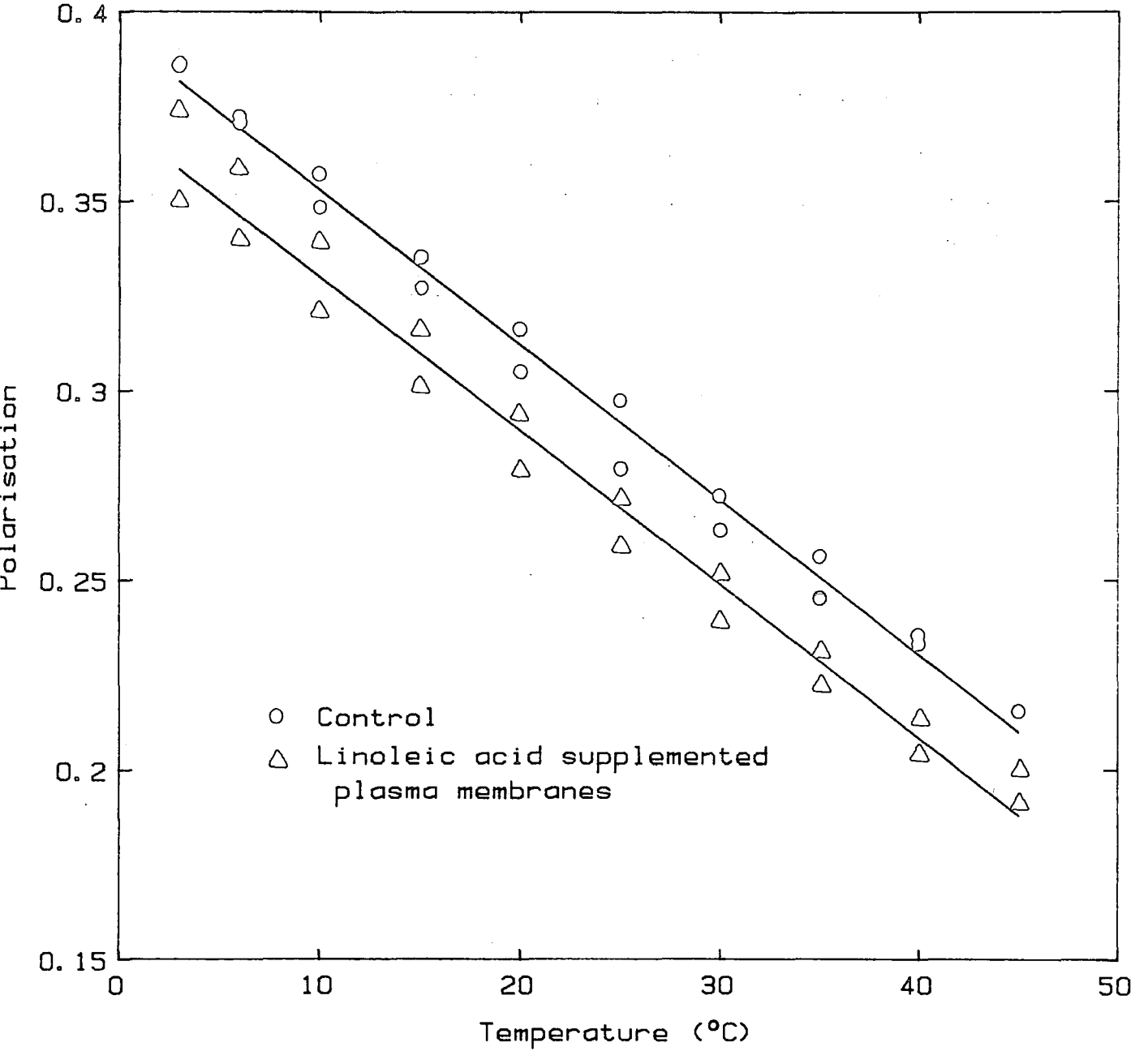

0.4
0.35
0.3
0.25
0.2
0.15
Polarisation
0
10
20
30
40
50
Temperature (°C)
Control
Linoleic acid supplemented plasma membranes

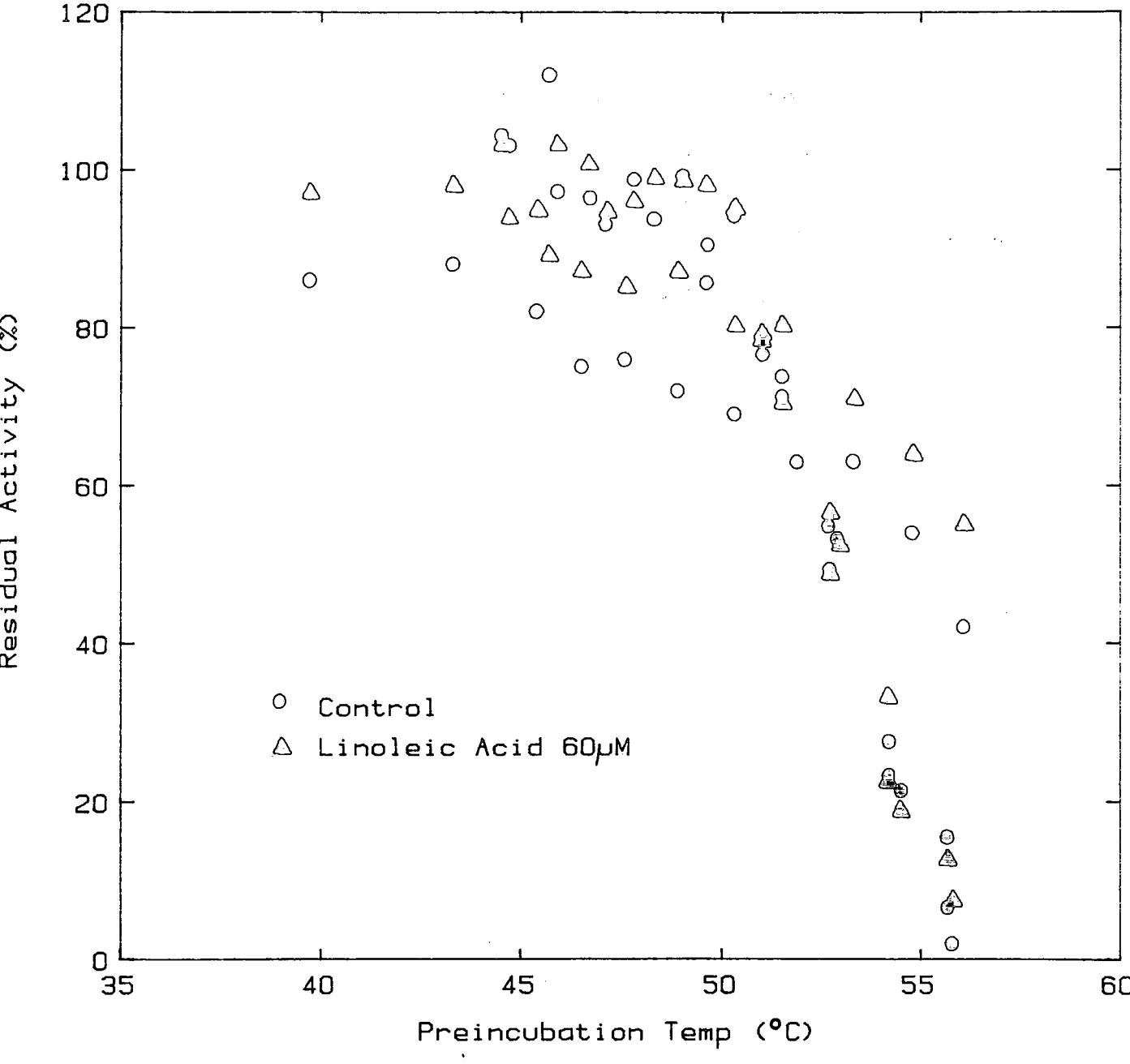
Figure 4.8

Thermal inactivation studies of the plasma membrane enzyme, Na^+/K^+ ATPase from control and linoleic acid supplemented HTC cells

Plasma membranes were purified by method 2, outlined in Materials and Methods, section 4.2.1(c)ii from control cells and from supplemented cells that had been exposed to $60\mu M$ linoleic acid for a 36 hour period.

Aliquots of purified plasma membranes were preincubated for 10 minutes at the temperatures shown on the graph and residual activity was assayed at $37°C$ as described in Materials and Methods, section 4.2.1(d)i.

Individual results derived from three separate experiments are shown on the figure.


120
100
80
60
40
20
0
Residual Activity (%)
35
40
45
50
55
60
Preincubation Temp (°C)
Control
Linoleic Acid 60μM

dose response curve, does not detect any difference in the thermosensitivity of this enzyme from the two membrane sources.

Isothermal inactivation studies offer a more sensitive means of studying the effect of supplementation procedures on membrane enzyme activity. The small quantities of purified plasma membrane material available limited such studies to a consideration of one membrane enzyme. Alkaline phosphodiesterase I as opposed to Na^+/K^+ATPase was selected for study as this analysis required less purified plasma membrane than the corresponding analysis of Na^+/K^+ATPase activity. Figure 4.9 shows typical results of isothermal inactivation studies conducted on the plasma membrane enzyme alkaline phosphodiesterase I, in purified plasma membranes isolated from control cells and from cells supplemented with $60\mu M$ linoleic acid for 36 hours. It is evident that this enzyme displays a curvilinear time-course during isothermal inactivation with a linear rate of inactivation at times greater than 13 minutes. It is thought that this inactivation profile represents a sequential decay of a thermolabile species of the enzyme into a more thermostable species.

$$e.g.\quad Thermolabile \xrightarrow{K_1} Thermostable \xrightarrow{K_2} Inactive\ species$$

where K_1 and K_2 are the first order decay constants with $K_1 > K_2$.

The slope of the final decay process defines K_2, whilst the decay constant K_1 for the thermolabile species can be derived by calculation as shown in the legend of figure 4.9. In the current study, it can be seen that whilst the K_2 values obtained in each experiment were very similar to each other, the K_1 values for control and supplemented cell membrane enzymes were quite different in 2 of the 3 experiments. Experimental data from 2 of the experiments suggests that the K_1 values were higher and the half life values shorter in the case of membranes derived from linoleic acid supplemented cells. However, because the third experiment showed very little difference in the values of K_1 and the half lives of this alkaline phosphodiesterase I enzyme from control and supplemented cells, the data is not found to be significant at the $p = 0.05$ level when paired

Figure 4.9

Isothermal inactivation studies of the plasma membrane enzyme alkaline phosphodiesterase I from control and linoleic acid supplemented HTC cells

Plasma membranes were purified by method 2, outlined in Materials and Methods, section 4.2.1(c)ii from control cells and from supplemented cells that had been exposed to $60\mu M$ linoleic acid for a 36 hour period. Aliquots of purified plasma membranes were preincubated at $64°C$ ($\pm 0.1°C$) for the times shown and the residual activity assayed at $37°C$ as described in Materials and Methods, section 4.2.1(d)ii.

The figure shows a typical set of data which indicates a sequential decay of a thermolabile species of the enzyme into a more thermostable species.

K_2, the decay constant of the thermostable species is defined as the gradient of the slope of the final decay process produced by plotting the natural logarithm of the enzyme activity versus exposure time.

K_1, the decay constant for the thermolabile species is defined as the gradient of the slope of the initial decay process shown in figure 4.9 and is calculated as the slope produced by plotting the activity of the thermolabile species (z) versus the exposure time (inset in figure 4.9).

The activity (z) of the thermolabile species is calculated as follows:

$$z = log_e((total\ activity) - (e^x.e^{-k_2 t}))$$

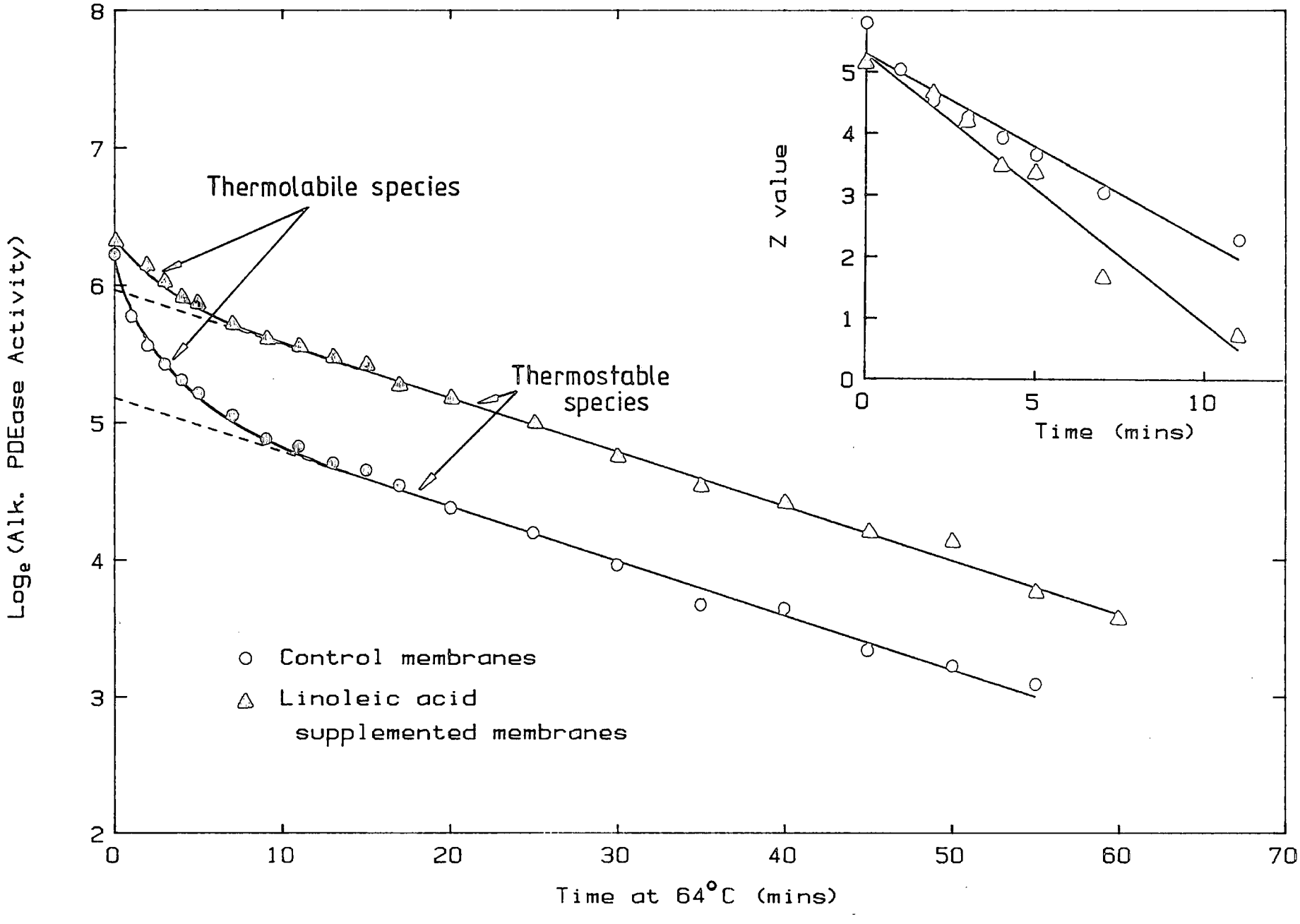
where x is intercept of the extrapolated decay of the thermostable species on the enzyme activity axis and t is the exposure time.

Parameters describing the thermal decay of alkaline phosphodiesterase I at $64°C$ from 3 separate experiments are given below.

Experiment	Control Membranes		
	k_1 $(\times 10^{-1}\ min^{-1})$	half life* (min)	k_2 $(\times 10^{-2}\ min^{-1})$
1	5.56	1.25	6.1
2	4.45	1.56	4.4
3	3.02	2.23	4.0
Experiment	Supplemented Membranes		
	k_1 $(\times 10^{-1}\ min^{-1})$	half life* (min)	k_2 $(\times 10^{-2}\ min^{-1})$
1	6.91	1.00	6.0
2	4.36	1.59	4.3
3	4.36	1.59	3.9

* half life is given by:

$$half\ life = \frac{ln2}{k_1}$$


Thermolabile species
Thermostable species
Control membranes
Linoleic acid supplemented membranes
Log$_e$ (Alk. PDEase Activity)
Time at 64°C (mins)
Z value
Time (mins)

t-tests are performed. Further studies would therefore be required to confirm the significance of this data.

4.4 Discussion

This chapter has described a new isolation procedure for HTC plasma membranes based on the use of self-forming Percoll gradients as compared to the sucrose density gradients used by a number of other workers (Lopez-Saura et al., 1978; Sauvage et al., 1981). Of the two methods tested in this study on the basis of the Na^+/K^+ATPase results it can be seen that the second method seems preferable as it produces plasma membranes of greater purity (approximately 1.2-fold) with only a slight reduction in yield as compared to the first method. Unfortunately, because of the number of markers being followed in method 2, a complete characterisation of all the fractions produced in the procedure was not possible. Consideration of such fractions in the future may well reveal ways of enhancing the purification and yield of plasma membranes even further. For example, Chakravarthy et al. (1985) working with cultured neuroblastoma cells, found that yields of plasma membranes could be greatly increased by careful adjustment of the pH and $MgCl_2$ concentration of the Percoll gradient. It seems that a higher pH serves to prevent clumping of subcellular organelles (Record et al., 1982) thereby improving the resolution of membranes on the gradient. This finding could in part explain the high loss of activity seen on the first Percoll gradient in method 1.

Clearly, the degree of purity and the yield of plasma membrane for any given cell type will depend on the nature of the procedure used to produce the plasma membrane and it would also appear to depend on the component used as the plasma membrane marker. For example, in the case of HTC cells the method reported by Tweto et al. (1976) involving a sucrose gradient separation only gave a 1.2% plasma membrane yield, purified 12-fold as compared to the original homogenate based on the assay of the marker enzyme 5′nucleotidase, whilst Sauvage et al (1981) using sucrose density gradients and digitonin, a complex polymer known to increase the buoyant density of the plasma membranes by binding to cholesterol, reported yields of HTC plasma membrane of 22.8% which were purified 21-fold as compared to the original homogenate based on the assay

of alkaline phosphodiesterase I. Although this purification scheme of Sauvage et al. (1981) produced highly purified plasma membranes it could not be employed in the current study since digitonin, being a membrane active agent, serves to disrupt the physical structure of the membrane, making it unsuitable if any physical measurements are to be carried out as was the intention in the current work.

Since Na^+/K^+ATPase is considered as one of the most reliable plasma membrane markers (Evans, 1982), it served as the principal plasma membrane marker in the current study. Based on the Na^+/K^+ATPase assay, method 1 in the present work gave a 36.5% plasma membrane yield purified 12.5-fold whilst method 2 gave a 28.5% plasma membrane yield purified 15.1-fold. Unfortunately, none of the available plasma membrane studies on HTC cells have utilised Na^+/K^+ATPase as a major plasma membrane marker. One of the reasons for this could be that although Na^+/K^+ATPase is considered as one of the most reliable plasma membrane markers, it is a transmembrane protein and requires access of ATP to the cytoplasmic face and ions to both faces of the membrane, which is often prevented during plasma membrane isolation procedures due to the formation of closed vesicles (Forbush, 1983). Thus the amount of Na^+/K^+ATPase activity measured will depend not only on the amount of enzyme present but also on the degree to which Mg^{2+}, ATP, Na^+ and K^+ are accessible to the appropriate sides of the membrane. In the current study a greater extent of vesicle formation in the homogenate may have led to an underestimate of the specific activity of this enzyme suggesting higher purification figures and yields than should actually have been the case. Due to the limited time available this possibility was not examined though any future studies should investigate this possibility by employing detergents to break open any vesicles that may have formed.

Clearly, further studies need to be conducted with adenylate cyclase, an enzyme which like Na^+/K^+ATPase is widely recognized as a reliable plasma membrane marker. However, if the present result is representative then it would certainly serve to support the Na^+/K^+ATPase results. Once again, HTC plasma membrane studies have not utilised adenylate cyclase as a marker enzyme though its existence in a number of rat and mouse liver and hepatoma plasma membranes has been demonstrated by a number of workers including Emmelot and Bos

(1971) and Makman (1971). These workers have shown that this enzyme is activated by various hormones according to cell type and to varying extents by different hormones.

Whilst the data for Na^+/K^+ATPase and adenylate cyclase show that plasma membranes were recovered by method 2 in high yield (approximately 20-30%) and were purified approximately 12 to 16-fold, the data for alkaline phosphodiesterase I and cholesterol suggest a much lower yield (10-13%) and purification (5 to 6-fold). Other workers have reported much higher purification figures for alkaline phosphodiesterase I. For example, Sauvage et al. (1981) working with HTC cells quoted a purification figure of 21-fold on a protein basis, whilst Loten and Loten-Redshaw (1986) working with rat liver plasma membranes reported a 26 fold purification based on this enzyme.

The precise reasons for the low purification values obtained with alkaline phosphodiesterase I are not clear. It could be that the parts of the plasma membrane containing the enzyme are being lost during this purification process, for although markers should be ideally distributed evenly within a single sub-cellular component there is increasing evidence for the heterogeneity of plasma membranes and the existence of membrane domains. A number of workers have separated liver plasma membranes into two or three sub-fractions. Evans (1970) found that his light subfraction contained most of the Mg^{2+}ATPase, leucine aminopeptidase and 5′nucleotidase, whilst the heavy fraction contained most of the Na^+/K^+ATPase. Haeffner et al. (1980) obtained two plasma membrane fractions from Ehrlich-Lettre ascites cells which showed variations in the levels of marker enzyme activity present. Thus it could well be that alkaline phosphodiesterase I exists in a different part of the HTC cell membrane to other plasma membrane markers from which it is separated during the isolation procedure.

Another possibility is that alkaline phosphodiesterase I is not in fact an ideal plasma membrane marker. Draye et al. (1987) have reported that as much as 10.9%-14.3% of alkaline phosphodiesterase I activity of a rat fibroblast cell homogenate was associated with a highly purified preparation of lysosomes. Similarly, Sauvage et al. (1981) have suggested that alkaline phosphodiesterase I in HTC cells in addition to having a plasma membrane location may also exist

within the membranes of endocytic vesicles, a finding based on a 30% latency in activity of this enzyme observed in cell homogenates.

Alternatively, the low values obtained with the alkaline phosphodiesterase I enzyme could simply reflect a lower stability of the enzyme during the isolation procedure as compared to the Na^+/K^+ATPase, or perhaps a decrease in the strength of its association with the membrane since these are factors that can also affect the reliability of enzymic determinations (Tsai et al., 1975).

The fact that comparable levels of purification were obtained with cholesterol and alkaline phosphodiesterase I agrees with the studies of Lopez-Saura et al. (1978) who found that cholesterol remained in close association with alkaline phosphodiesterase I during the purification process in HTC cells. The level of cholesterol in the cell homogenate and plasma membranes of HTC cells in the current study was 0.029 and 0.186 μmoles/mg protein respectively. These values are generally much lower than those given by other workers. For example, Koizumi et al. (1981) give cholesterol values for L-1210 cell homogenates and 23-fold purified (based on the assay of Na^+/K^+ATPase) plasma membranes of 0.044 and 0.347 μmoles/mg protein using ferric chloride reagent by the method of Courchaine et al. (1959). Part of the reason for the lower values in the present method however, could well be due to the cholesterol assay procedure used, for whilst the Johnson (1979) method employed in the current study gives results in agreement with those from gas chromatography, many of the older, less specific analytical methods for cholesterol such as those involving the use of ferric chloride have been shown by Johnson to give cholesterol results which are 10 to 33% higher.

Once again the suitability of cholesterol as a plasma membrane marker is questionable since it is also known to occur at lower concentrations in a number of intra-cellular membranes. For example, Amar-Costesec et al. (1974) have suggested its association with the Golgi apparatus. Henning and Heidrich (1974) have shown the rat lysosomal plasma membrane to be rich in cholesterol. Other workers have found the results produced with cholesterol at variance with those produced by other putative plasma membrane markers. For example, Payrastre et al. (1988) observed a 7.7-fold enrichment in cholesterol content of human

epidermoid carcinoma cell purified plasma membrane over the cell lysate. However, binding of $[^3H]$ concanavalin A, which is considered to be a good probe of the external cell surface (Record et al., 1982), and 5′ nucleotidase activity appeared to be enriched in the purified plasma membrane only 4.4-fold and 4.5-fold respectively over the cell lysate.

In any study concerned with the production of plasma membranes in a purified state it is obviously important to monitor the level of contamination of the final plasma membrane fraction with other subcellular organelle material. Because time was a limiting factor, in this study it was only possible to consider levels of contamination produced by the most likely contaminants, namely, endoplasmic reticulum, mitochondria and lysosmal material. The greatest source of these contaminants in the final plasma membrane fraction produced by the isolation procedure of method 2 came from the endoplasmic reticulum, with the least contamination from mitochondria. Yields of endoplasmic reticulum, lysosomal and mitochondrial marker enzymes in the current study were 2.81%, 1.57% and 0.63% respectively with corresponding purification figures of 1.76-fold, 0.97-fold and 0.39-fold. Chakravarthy et al. (1985) using a similar Percoll purification process but with a different cell line (neuroblastoma cells) also reported a much higher yield of endoplasmic reticulum marker (7.6%) than mitochondrial marker (1.5%) with purification figures of 1.4-fold and 0.3-fold respectively as compared to the original homogenate. Lopez-Saura et al. (1978) carried out an analytical fractionation of HTC cells producing four particulate fractions and a supernatant by a differential centrifugation procedure. Results were expressed as a percentage of the total activity of a component recovered in the five fractions. It is interesting to observe that the particulate fraction (P) in their study found to contain the highest level (45.6%) of plasma membrane material, assayed using the marker enzyme alkaline phosphodiesterase I, was also characterised by very high levels of NADPH-cytochrome *c* reductase (53.7%) and lower levels of N-acetyl β-glucosamidase (12.2%). This indicates that alkaline phosphodiesterase I was very poorly resolved from enzyme markers of other organelles, especially NADPH-cytochrome *c* reductase. In a later preparative procedure designed by Lopez-Saura and his co-workers (Sauvage et al., 1981) the original differential centrifugation procedure (Lopez-Saura et al., 1978) was repeated in the presence of small quantities of digitonin. This procedure resulted in the production of a

plasma membrane fraction containing 22.8% alkaline phosphodiesterase I which was purified 21-fold and contained only 1.2% NADPH-cytochrome *c* reductase purified 1.09-fold and 2.0% N-acetyl β-glucosaminidase purified 1.8-fold. These results of Sauvage et al. (1981) indicate a slightly better purification figure for N-acetyl β-glucosaminidase than the current study.

NADPH-cytochrome *c* reductase was used as the enzymic marker for endoplasmic reticulum since work by Lopez-Saura et al. (1978) suggests that HTC cells do not contain 'typical' glucose-6-phosphatase. 'Typical' glucose-6-phosphatase exhibits a number of properties including inactivation following preincubation without glucose-6-phosphate as a substrate (De Duve et al., 1949), not found in HTC cells. The absence of typical glucose-6-phosphatase activity in HTC cells, which has also been observed in a number of other hepatomas (Morris, 1965) is thought to result from their dedifferentiated sate. Lopez-Saura et al. (1978) reported a specific activity for NADPH-cytochrome *c* reductase in HTC cell homogenates of 0.0137 μmoles cytochrome *c* reduced/*mg* protein/*min*. The slightly higher figure of 0.0195 obtained in the present study is thought, in part, to reflect the increased level of Triton X-100 that was used, 1% (*w*/*v*) final concentration as opposed to 0.1% (*w*/*v*), which was found to enhance the linearity of the reaction rate with a slight increase in the reaction rate measured. Loten and Redshaw-Loten (1986) working with rat liver reported a specific activity for this enzyme in homogenates of 0.0175 and a plasma membrane activity of 0.0033, giving a 0.18-fold purification figure and a 0.1% yield. This represents an improvement in their purification process with respect to the current method since initial levels of specific activity recorded by both methods are comparable. Reports on plasma membrane purifications from murine L1210 cells by Tsai et al. (1975) and Koizumi et al. (1981) indicate much lower specific activities of NADPH-cytochrome *c* reductase for homogenates of 0.0037 and 0.0085 respectively and for purified plasma membrane fractions of 0.0019 and 0.0038 with purification values of 0.5-fold and 0.44-fold again suggesting a lower contamination of the final plasma membrane fraction with endoplasmic reticulum than is the case in the current study. However, Sun et al. (1988) working with rat cerebral cortex reported a 2.5-fold purification of NADPH-cytochrome *c* reductase in plasma membrane preparations of glial and neuronal soma cells which is higher than the 1.76-fold purification figure and hence level of contamination obtained

with NADPH-cytochrome *c* reductase in the present study. These varied results suggest that the level of endoplasmic reticulum contamination in any membrane preparation depends not only on the type of separation technique employed but also on the cell source.

The N-acetyl β-glucosaminidase measured as the marker for lysosomal contamination showed the latency typical of lysosomal enzymes reported by Lopez-Saura et al. (1978) and the assay for this enzyme was consequently performed in the presence of 0.1% (w/v) Triton X-100. Whilst the 1.57% yield of N-acetyl β-glucosaminidase obtained in this study was comparable to the 2% yield reported by Sauvage et al. (1981) also working with HTC cells, the 0.97-fold purification figure reported in this study represents an improvement over their 1.8-fold purification figure but is not as low as the 0.43-fold purification figure reported for this enzyme in a plasma membrane fraction purified from the human epidermoid carcinoma cell line A431 (Payastre et al., 1988).

Most workers report a very low yield of mitochondrial contamination in purified plasma membranes. Tsai et al. (1975) working with murine L1210 cells reported a specific activity of succinate dehydrogenase in cell homogenates of 0.05 μmoles cytochrome *c* reduced/mg protein/min, a figure much higher than the 0.0122 figure recorded for this enzyme in the current study. However, they did not detect this enzyme in the final putative plasma membrane fraction suggesting an improved purification process relative to the current study. Koizumi et al. (1981) on the other hand, also using L1210 cells reported a specific activity of succinate dehydrogenase in cell homogenates of 0.03 and in final membranes of 0.018 giving a 0.6-fold purification figure that was slightly higher than the 0.394-fold figure obtained in the current study, suggesting a higher contamination of the plasma membrane with mitochondrial material than was the case in the present purification procedure. Also, whilst the 0.394-fold purification of succinate dehydrogenase in the current study is comparable to the 0.3-fold purification figure obtained by Chakravarthy et al. (1985) in their study with neuroblastoma cells, the lower yield (0.63%) of mitochondrial material in the current study represents an improvement over the 1.5% yield reported by Chakravarthy et al. (1985).

It is evident that method 2 described in the present chapter for the isolation

of HTC cell plasma membranes has provided a rapid and reliable method for producing plasma membranes in high yield and with similar levels of purity from cell suspensions of both normal and linoleic acid supplemented HTC cells. The establishment of a suitable procedure for the isolation of a relatively pure plasma membrane fraction has important consequences as it enables further comparative studies to be made between control and fatty acid supplemented cells at the level of the plasma membrane. For example, from the results it would appear that supplementation of HTC cells grown in Eagles Minimum Essential Medium with $60\mu M$ linoleic acid in the presence of newborn bovine serum (10%, v/v) can produce modifications in the fatty acid composition of the HTC cell plasma membranes (Table 4.5). These modifications do not appear to be accompanied by a change in membrane total phospholipid or cholesterol content (Table 4.6) but do appear to be associated with an increase in membrane 'fluidity' (figure 4.7) as measured by DPH fluorescence polarisation.

Although many other workers have shown that fatty acid supplementation procedures can be used to alter the lipid composition of cells, and membrane related properties such as enzymatic activity (Poon et al., 1981) and fluidity, there is a lack of uniformity in the level of the analysis. Differences in method exist not only in the means and period of supplementation but also in the fractionation of the tissue and the subsequent separation of the lipids which makes comparisons with the present study difficult. For example, Ferguson et al. (1975) working with mouse LM cells showed that supplementation with $17\mu M$ linoleic acid for a 16-24 hour period produced only trace amounts ($< 2\%$) of its long chain elongation and desaturation products 20 : 2 and 20 : 3 in total LM cell phospholipids, whilst after a 48 hour exposure to the same concentration of linoleate these two metabolic products of linoleic acid constituted 38% and 5.5% of the phospholipid fatty acid composition respectively. Similarly, as suggested in the introduction, whilst analysis of total cellular phospholipids may give an indication of the direction of plasma membrane alterations, very often it will not reflect the extent of such changes (Burns et al., 1983). Clearly, in the current study, since a suitable plasma membrane isolation procedure has been developed enabling investigations to be conducted at the level of the plasma membrane, the most useful comparisons are to be drawn from other plasma membrane based

studies and from studies involving HTC cells, since different cell lines may show variations in their ability to metabolize exogenously supplied fatty acids.

Wood (1973) has examined the effect of serum lipid levels on the composition of cellular lipids in HTC cells. A range of media were prepared containing decreasing amounts of bovine and/or fetal calf serum. Separate media were also prepared containing lipid free fetal calf serum. He found that although some differences were noted, in general cells grown in different media had comparable phospholipid class and fatty acid compositions. i.e. the fatty acid composition of total phospholipids derived from cells grown in medium containing 20% bovine serum and 5% fetal calf serum was very similar to that of cells grown in medium containing 5% lipid-free fetal calf serum. These findings led Wood to suggest that exogeneous serum lipids may have less influence on HTC cell lipid biosynthesis than is the case for most other cultured cells.

The presence or absence of desaturation and/or elongation enzymes in mammalian cells can lead to considerable variation not only in the fatty acid profiles of cellular phospholipids in control cells but also in the extent to which different fatty acids can be increased in cells by supplementation. In the present study, supplementation of HTC cells with linoleic acid did not produce an increase in its metabolic product 20 : 4. This agrees with the findings of Spector et al. (1979) working with human skin fibroblasts, and Needleman et al. (1982) working with human platelets. On the other hand, studies by Denning et al. (1982) with 3T3 mouse fibroblasts and by Hyman and Spector (1981) with Y79 retinoblastoma cells have shown that these particular cells become enriched with 20 : 4 when they are supplemented with linoleic acid. This difference is thought to relate to different levels of activity of the $\Delta 6$ and $\Delta 5$ desaturase enzymes involved in the conversion of linoleic to arachidonic acid.

A large number of lipid metabolism studies have been conducted on HTC cells. It has been shown that HTC cells retain the biosynthetic capacity to desaturate and elongate fatty acids. For example, Alaniz et al. (1975) have shown that cultured HTC cells retain the ability to desaturate stearic to oleic acid ($\Delta 9$ desaturase), α-linolenic acid to octadeca-6,9,12,15-tetraenoic acid ($\Delta 6$ desaturase) and eicosa-8,11,14-trienoic acid to arachidonic acid ($\Delta 5$ desaturase).

The low level of arachidonic acid noted in the plasma membrane in the current study (Table 4.5) has been observed at the cellular level (Alaniz et al., 1975) and has been shown to be related to the diminished capacity of HTC cells to synthesise this acid from linoleic acid. Studies have indicated that whilst α-linolenic acid is rapidly converted to eicosa-5,8,11,14,17-pentaenoic acid, exogenous linoleic acid that is a precursor of eicosa-8,11,14-trienoic acid is converted to arachidonic acid only in very small amounts (Alaniz et al., 1975). The difficulty of cells in converting exogeneous linoleic acid to arachidonic acid is not due to the absence or low activity of $\Delta 5$ desaturase since Gaspar et al. (1975) have shown that the conversion of eicosa-8,11,14-trienoic acid to arachidonic acid, that requires $\Delta 5$ desaturase, readily takes place. Dunbar and Bailey (1975) confirmed these results and showed that a series of heteroploid cells were not characterised by a $\Delta 6$ desaturase enzyme since they were unable to desaturate linoleic acid, but were characterised by a $\Delta 5$ desaturase enzyme since they were able to desaturate eicosa-8,11,14-trienoic acid. They concluded that the cells suffered a loss or modification of the $\Delta 6$ desaturase enzyme. However, since HTC cells do show an active $\Delta 6$ desaturation of α-linolenic acid and since it is thought that the same $\Delta 6$ desaturase desaturates oleic, linoleic and α-linolenic acid (Ninno et al., 1974), Gaspar et al. (1975) have suggested that HTC cells are characterised by a highly specific inhibition of the $\Delta 6$ desaturase. This inhibition is envisaged only to decrease linoleic acid desaturation to eicosa-8,11,14-trienoic acid and to have no effect on α-linolenic conversion to octadeca-6,9,12,15-tetraenoic acid.

Other studies with HTC cells at the cellular level have revealed that in addition to the well established pathways for the synthesis of polyunsaturated fatty acids from α-linolenic and linoleic acid (figure 4.1) HTC cells have other biosynthetic pathways that utilise these two essential fatty acids (Alaniz et al., 1976). Whilst both these fatty acids form a substrate for a $\Delta 6$ desaturase they have also been found to form the substrate for an elongation reaction. Linoleic acid elongation leads to $20:2\ (\Delta 11,14)$ which is then desaturated by $\Delta 5$ desaturase to $20:3\ (\Delta 5,11,14)$, whilst α-linolenic acid is elongated to $20:3\ (\Delta 11,14,17)$ which is then desaturated again by $\Delta 5$ desaturase to $20:4\ (\Delta 5,11,14,17)$. (The Δ notation indicates the position of the double bond from the carboxyl end of the molecule).

Mahler et al. (1988) have isolated plasma membranes from liver and HTC cells, grown as the Morris hepatoma 7288C in the hind legs of male Buffalo rats, and examined the phospholipid acyl groups. Whilst the trend in fatty acyl composition of the HTC cell plasma membrane phospholipids agrees with the findings of the current study, the level of oleic acid in Mahler's study was much lower, at 27% of total fatty acyl groups as opposed to 40% in the present investigations. This difference may well be due to the different dietary conditions experienced by the cells due to the method of growth, i.e. *in vivo* tumour as opposed to monolayer culture.

The elevation in linoleic acid that occurs in the plasma membrane phospholipid of HTC cells following exposure of cells to linoleic acid supplemented medium has been reported by a number of other workers employing a variety of cell lines. King and Spector (1978) working with Ehrlich ascites cells found that the plasma membrane phospholipids were enriched with 18 : 2 when cells were exposed to this fatty acid bound to albumin during a 4 hour incubation period. In this study by King and Spector (1978) membranes isolated from cells exposed to 18 : 1 served as the point of reference for the comparison of fatty acyl enrichments. It was found that the membrane fraction of cells exposed to 18 : 1 contained 39% 18 : 1 and only 5% 18 : 2 whilst the membrane fraction isolated from cells exposed to 18 : 2 contained 31% 18 : 1 and 12% 18 : 2. In keeping with the current study there was no appreciable change in the cholesterol/phospholpid ratios of supplemented cells relative to control cells.

Poon et al. (1981) working with murine T lymphocyte EL4 tumour cells reported an increase in the level of 18 : 2 in plasma membrane phospholipids from 12% in control cells to 40.7% in supplemented cells following a 24 hour exposure of the cells to $115\mu M$ linoleic acid.

Sweet and Schroeder (1988) working with mouse LM fibroblasts which were cultured in suspension for 3 days in lipid-free medium or identical medium containing $17\mu M$ linoleate (18 : 2) bound to bovine serum albumin, showed that the plasma membranes from cells cultured in lipid free medium contained only saturated and monosaturated fatty acids. Palmitic (16 : 0) and oleic (18 : 1) acids together comprised 76% of the plasma membrane phospholipid acyl groups. Sup-

plementation with $17\mu M$ linoleic acid as described above, however resulted in the incorporation of 5% linoleic acid into membranes with a concomitant reduction in palmitic acid from 21% to 8%. Once again, this supplementation procedure did not alter the sterol/phospholipid molar ratio values of the plasma membrane which were 0.74 ± 0.09 and 0.60 ± 0.10 in plasma membranes from control and linoleate supplemented cells respectively.

Edwards-Webb and Gurr (1988) showed that when guinea pigs were fed diets containing a high proportion of linoleic acid, (31% by molar proportion of the fatty acid present), the fatty acid composition of the lipids of the erythrocyte membrane was enriched in linoleic acid from 15% in control membranes to 21% in supplemented membranes. However, unlike the current study, these workers reported that membranes enriched in linoleic acid also contained a higher cholesterol/phospholipid ratio.

The finding that the proportion of saturated fatty acid in the plasma membrane remained fairly constant at approximately 50% of total fatty acyl groups (figure 4.5) has also been reported by a number of other workers. It seems that biological mechanisms that detect changes in the fatty acyl content of membrane phospholipids are particularly sensitive to the ratio of saturated:unsaturated fatty acids. In addition it seems that the polyunsaturated fatty acid content of tissues is regulated by substitutions almost entirely among unsaturated fatty acids (Stubbs and Smith, 1984). A number of supplementation studies at the membrane level have revealed that the relative amounts of saturated and unsaturated fatty acids cannot be changed much from 1 : 1, although a great deal of unsaturated fatty acid interchanging can occur. For example, Neudoerffer and Lea (1967) working with sarcoplasmic reticulum from turkey breast found that enriching the diet with highly unsaturated fatty acids caused a marked change in the unsaturated fatty acid content of the membrane but little change in the saturated fatty acids. In particular, the content of the polyunsaturated fatty acids 20 : 5 and 22 : 6 were increased at the expense of 18 : 1 and 18 : 2. This constancy in total saturated fatty acids no doubt relates to a considerable degree to the specificity of enzymes such as phospholipase A_2 for the $sn-1$ and $sn-2$ positions in phospholipids and in the segregation of saturated and unsaturated

fatty acids between those positions which invariably occurs (Lands and Hart, 1966).

Cossins et al. (1986) have suggested that relationships between lipid biosynthesis, lipid composition, membrane order and membrane function are often best revealed by correlating changes produced in one by experimental treatment with changes produced in another. In the current study, supplementation of HTC cells with linoleic acid produced no significant change in the cholesterol/phospholipid molar ratio, a marked alteration in the phospholipid fatty acid composition and a decrease in steady state fluorescence polarisation values of plasma membranes. Such results suggest that the decrease in polarisation values observed in supplemented cell plasma membranes could well be correlated to the changes in the fatty acyl composition of the membrane phospholipids.

A number of other workers have reported changes in membrane 'fluidity' measurements as a result of supplementation procedures. Wolters and Konings (1984) working with mouse fibroblast LM cells showed that when these cells were grown in a serum-free medium supplemented with $100\mu M$ arachidonic acid (20 : 4) complexed to bovine serum albumin for a 24 hour period, the percentage of polyunsaturated fatty acyl chains in membrane phospholipids increased from 6.8% to 44.3% whilst a decrease in polarisation values was observed. George et al. (1983) cultured human lymphoid LDV cells in medium containing delipidated serum supplemented with either $40\mu M$ oleic (18 : 1) or $40\mu M$ linoleic (18 : 2) acid for periods of time between 3 and 120 days. At a cellular level they showed that after 3 or more days growth, not only were there substantial increases in the proportion of oleate and linoleate present in the cellular lipid respectively, but also that there was an increase in membrane fluidity as measured by DPH. This increase in membrane fluidity was found to be greater for cells grown in the presence of linoleic acid than for cells grown in the presence of oleic acid.

Storch and Schachter (1984) working with rat hepatocytes showed that when the content of monoenoic and polyenoic acyl chains in plasma membrane lipids was increased *in vivo* by a dietary regimen involving intermittent starvation followed by refeeding with a fat-free diet, the fluidity of the plasma membranes, which was measured by steady state fluorescence polarisation of DPH, DL-2-

(9-anthroyloxy) stearate (2AS) and DL-12-(9-anthroyloxy) stearate (12 AS), was significantly increased. There was also a decrease in the cholesterol/phospholipid molar ratio suggesting that the increase in fluidity was the result of more than one factor in this instance.

Alam and Alam (1986) working with plasma membranes of rat submandibular salivary glands reported a decrease in fluidity of membranes following exposure of rats to diets deficient in essential fatty acids. Whilst this decrease in membrane fluidity was accompanied by a decrease in the double bond index (which is a measure of the degree of unsaturation of lipids) relative to control membranes, there was no change in the cholesterol/phospholipid molar ratio.

However, an association between membrane fatty acid composition changes and membrane fluidity changes does not occur in all systems. Poon et al. (1981) showed that significant alterations in the saturated and unsaturated fatty acyl composition of the membrane phospholipids of murine T lymphocyte EL4 tumour cells could be achieved by exposing cells to fatty acids for 24 hour periods. For example, incubation of the cells with $60\mu M$ oleic acid (18 : 1) resulted in a 20% increase in the level of 18 : 1 in the plasma membrane phospholipids. However, there were no significant differences in 'fluidity' as measured by Electron Spin Resonance (ESR) spectroscopy that were recorded suggesting that the T-lymphocyte must have a compensatory mechanism enabling it to resist changes in membrane fluidity. A similar finding was reported by Edwards-Webb and Gurr (1988) who modified the fatty acid composition of the plasma membrane phospholipids of guinea pig erythrocytes by feeding guinea pigs diets rich in linoleic acid. Whilst the plasma membrane phospholipids were found to be enriched in linoleic acid, the 'order' parameter of the membrane, as determined by ESR spectroscopy was unaffected. Further studies revealed a higher ratio of cholesterol to phospholipid in these supplemented membranes. The findings of these investigations has led Edwards-Webb and Gurr (1988) to propose that the physical properties of erythrocyte membranes are normally maintained relatively constant in response to different dietary fats by compensatory adjustments of the proportions of both polyunsaturated fatty acids and cholesterol.

Clearly, in the current study with HTC cells it would appear that membrane

fatty acid compositional changes rather than changes in cholesterol/phospholipid levels are involved in determining membrane fluidity changes. However, it should not be assumed that membrane fluidity and the extent of fatty acid unsaturation are always related in the simple and direct manner suggested by the current results. As Stubbs and Smith (1984) have indicated, the relative proportion of fatty acid types do not always give predictable effects in terms of membrane physical properties. For example, it was thought that increasing the level of unsaturation of fatty acids would result in an increase in membrane fluidity due to the increased space occupied by their molecular structures. However, it has been shown by Van Deenen (1971) working with model systems, that whilst the introduction of the first and second double bond into a saturated fatty acid causes a marked effect on bilayer fluidity, further unsaturation has little effect on the fluidity. In addition, the position of double bonds along the hydrocarbon chain and chain length can have important effects in terms of membrane fluidity.

It is also important to bear in mind that biological membranes contain a considerable diversity of phospholipids. Each unique molecule, totally defined with respect to every functional group, represents a 'molecular species'. Such a definition includes the identity of each fatty acid, aldehyde or alcohol and the position in the molecule at which it is attached. The current study, in common with the majority of studies to date, has not been conducted at the level of the molecular species which is a disadvantage in any attempt to relate physical properties, function and composition since it means that misleading conclusions may be drawn. This was highlighted by Dickens and Thompson (1982) in their study of the response of lipids in *Tetrahymena pyriformis* to adjustment of the growth temperature. Initially it was thought that the rapid response of *Tetrahymena* to changes in the growth temperature was the result of changes in the fatty acyl group composition. However, analysis at the molecular species level has indicated the importance of the presence of ether linkages in the rapid response to temperature.

Again, it is possible that the supplementation procedures adopted in the current study may have produced additional perturbations to the plasma membrane structure which have not been investigated, with the result that other

mechanisms may be responsible for the fluidity changes observed. For example, phospholipid polar head group composition is known to be an important factor in bilayer fluidity. Whilst many of the supplementation studies with cultured cells (King and Spector, 1978; Spector et al., 1979) have not observed any changes in the phospholipid head group composition following supplementation, it is known that an increase in the ratio of phosphatidylethanolamine (PE) to phosphatidylcholine (PC) causes an increase in the steady state polarisation of DPH (Gilmore et al., 1979). The PC/PE ratio is particularly important since PE is a hydrogen donor and can form hydrogen bonds with the anionic phosphate-oxygen of the adjacent phospholipid thus restricting the movement of the acyl chains (Chapman and Wallach, 1968).

Clearly, whilst the increased levels of linoleic acid present in HTC plasma membranes, following supplementation with this particular fatty acid, would appear to produce a significant effect on membrane fluidity measurements, it is likely that in reality such changes that are observed result from an interrelationship of a number of factors. These include the level of unsaturation of the fatty acyl chains, the cholesterol content and the phospholipid head group composition within the plasma membrane.

The idea that a cell's response to hyperthermic insult may well be related to its membrane 'fluidity' at the time of treatment (Dennis and Yatvin, 1981; Mulcahy et al., 1981; Yatvin et al., 1987) and the fact that a few survival studies of normal and neoplastic cells in culture have suggested that cancer cells may be more sensitive to the lethal effects of hyperthermia than normal cells (Giovanella et al., 1973), makes it interesting to compare the DPH fluorescence polarisation measurements obtained in the current study with those of other workers.

Control membranes from HTC cells were characterised by a polarisation value of 0.240 at $37°C$ which compares favourably with the DPH polarisation value of 0.247 at $37°C$ recorded by Wolters and Konings (1984) working with mouse fibroblast LM cell membranes. Barker (1985) working with plasma membranes derived from rat liver cells and two transplantable rat tumours (MC7 and D23) recorded a higher DPH polarisation value of 0.290 for liver plasma membranes but comparable polarisation values of 0.250 and 0.230 for the MC7 and D23 tumour

cell plasma membranes respectively. Although relatively few studies have been carried out on tumour plasma membranes using the technique of steady state fluorescence polarisation spectroscopy, a number of those which have (e.g. Van Blitterswijk et al., 1987; Koizumi et al., 1981; Johnson, 1981) have revealed decreased DPH polarisation values in tumour cells. For example, Koizumi et al. (1981) working with plasma membranes from various mouse lymphoid cells recorded a range of DPH fluorescence polarisation values of 0.254-0.261 at $25°C$ for thymocyte plasma membranes and a lower range of DPH polarisation values of 0.235-0.241 for X-ray induced thymoma cell plasma membranes at the same temperature. Thus it could be that some tumour cells show an increase in thermal sensitivity relative to normal cells due to a difference in the plasma membrane 'fluidity' (order) between tumour and normal cells.

The decrease in polarisation value from 0.240 to 0.220 at $37°C$ observed with linoleic acid supplemented membranes in the current study which was accompanied with an increase in thermal sensitivity of intact cells also implicates the importance of 'fluidity' in the hyperthermic response of cells. A similar finding was reported by Wolters and Konings (1984) when mouse fibroblast LM cells were supplemented with $100\mu M$ arachidonic acid for a 24 hour period. A decrease in polarisation values from 0.247 to 0.223 was observed in purified plasma membranes together with an increase in the thermal sensitivity of the cells.

Membrane associated enzyme activity, as stated in the introduction, is often found to be influenced by the 'fluidity' (order) of membrane lipids (Kimelberg, 1977). Part of the reason for this influence undoubtedly stems from the anisotropic hydrophobic core of the membrane which forms a viscous, relatively hindered environment for enzyme fuctioning (Cossins et al., 1981). Since enzymes require molecular flexibility for catalytic purposes it is probable that such an environment will cause enzymes to adopt relatively loose tertiary structures which in turn are likely to be susceptible to perturbation. Any increase in membrane 'fluidity' caused for example by supplementation procedures and/or hyperthermic temperatures are likely to produce a less hindered environment for the proteins. This less ordered environment in turn is likely to permit a greater range of conformational movement which could lead to the adoption of inactivating configurations by membrane proteins.

In the current study where a 36 hour period of supplementation with $60\mu M$ linoleic acid had been found to increase the 'fluidity' (decrease the order) of the plasma membrane (figure 4.7), it was hoped that hyperthermic studies would reveal a marked increase in the thermal sensitivity of supplemented membrane proteins relative to control membrane proteins. Unfortunately the results of Na^+/K^+ATPase enzyme were inconclusive. However, the isothermal inactivation studies conducted with alkaline phosphodiesterase I seem to suggest that the enzyme from the supplemented cell plasma membranes was indeed more thermolabile than the same enzyme from control membranes.

Conflicting results have been obtained by other workers who have conducted hyperthermic studies on membrane enzymes. For example, Burdon et al. (1982, 1984) reported that the Na^+/K^+ATPase activity in HeLa cells was rapidly inactivated at $45°C$, though this activity was partially restored by subsequent incubation at $37°C$, if the hyperthermic treatment was brief (approximately 10 minutes). On the other hand, Stevenson et al. (1983) working with CHO cells and Boonstra et al. (1984) working with hepatoma H35 cells found an enhanced ouabain sensitive K^+ influx during hyperthermia at $42°C$ which was completely reversible when cells were returned to $37°C$. Similarly, Bates and Mackillop (1985) also working with CHO cells showed that ouabain sensitive $^{86}Rb^+$ influx increased with temperatures between $37°C$ and $45°C$ and was only irreversibly inhibited above $45°C$.

Bowler et al. (1973) suggested that cellular heat injury may well result from the thermal sensitivity of membrane lipoproteins on the basis of a number of *vitro* experiments. For example, 3 enzymes from crayfish muscle membrane; Mg^{2+}ATPase, Na^+/K^+ATPase and Ca^{2+}ATPase were found to be thermolabile in the range of temperatures lethal to the whole organism. In addition, pyruvate kinase, a soluble enzyme from the muscle, was found to be much more thermostable than the membrane-associated enzymes. However, the thermostability of the plasma membrane enzymes Na^+/K^+ATPase and Mg^{2+}ATPase, unlike heat death of the organism, was not modified by acclimation temperature in this instance. In contrast, Cossins et al. (1981) clearly demonstrated a resistance acclimation effect on the thermal sensitivity of goldfish synaptic membrane Na^+/K^+ATPase with the enzyme from $28°C$ acclimated fish being more

thermally resistant than that from $6°C$ acclimated fish. The thermal stability of goldfish brain synaptic membrane acetyl cholinesterase was similarly found to be dependent upon acclimation temperature (Bowler, 1987).

Barker (1985) working with the Mg^{2+}ATPase enzyme from rat liver and two transplantable rat tumours (MC7 and D23) found that the Mg^{2+}ATPase from the tumours was far more thermolabile than the same enzyme from liver and that the membrane lipid order as measured by DPH fluorescence polarisation correlated with the thermal sensitivity of the three enzymes, i.e. the less ordered (more fluid) the membrane lipid the greater the sensitivity of the enzyme to heat.

In summary, it is evident that development of a rapid and reliable method for the isolation of HTC cell plasma membranes in high yield and with a high degree of purity has enabled investigations into the effect of plasma membrane composition on the thermal sensitivity of these tumour cells to be conducted. Such studies in turn have revealed that changes do occur in terms of plasma membrane lipid composition and physical state when HTC cells are exposed to $60\mu M$ linoleic acid supplement for a 36 hour period. Since supplementation of HTC cells under these conditions has already been shown to be linked to an increase in the thermal sensitivity of these cells, the findings of the current chapter lend support to the idea that the plasma membrane is an important site of hyperthermic damage and would seem to implicate the 'fluidity' of the plasma membrane as being a key factor in determining the response of cells to heat treatment.

However, although this study and many others (e.g. Dennis and Yatvin, 1981; Mulcahy et al., 1981; Yatvin et al., 1987) have suggested that hyperthermic sensitivity is related to membrane fluidity, there are instances where 'fluidity' appears to have no effect on hyperthermic sensitivity. Studies by Lepock and coworkers (Lepock et al., 1981; Massicotte-Nolan et al., 1981) in particular have questioned the importance of membrane lipid fluidity in hyperthermic cell killing. Investigations conducted on V79 Chinese hamster lung fibroblasts cells with butylated hydroxytoluene (BHT), an effective lipid perturber (Lepock et al., 1981), indicated that BHT treatment which was found to increase membrane fluidity as measured by the Electron Spin Resonance (ESR) probe 2,2-dimethyl-5-dodecyl-

5-methyloxazolidine-N-oxide (2N14) did not sensitise cells to hyperthermia nor was there any decrease in membrane fluidity observed in thermotolerant V79 cells compared to control cells. Similarly, Lepock and coworkers (Massicotte-Nolan et al., 1981) again working with V79 Chinese hamster lung fibroblast cells looked at the effects of short chain alcohols including methanol, ethanol and 2-propanol on the hyperthermic response of cells. In this particular study these workers reported that the membrane fluidising ability of these alcohols as measured by the ESR probe 2N14 did not correlate well with the degree of heat sensitisation by the same alcohols. However, a relationship was found to exist between the ability of monohydric, dihydric and trihydric alcohols to sensitise or protect proteins from heat denaturation and their ability to sensitise or protect cells from heat. These findings led Lepock and coworkers to suggest that protein denaturation was the rate-limiting step in hyperthermic cell killing (Massicotte-Nolan et al., 1981).

In 1982 Lepock (Lepock, 1982) suggested that part of the reason for the discrepancies that were being reported in the importance of 'fluidity' in hyperthermic cell killing could result from the poor definition and hence measurement of 'fluidity'. Whilst lipid motion and lipid order and often less well defined parameters were usually classified together as membrane 'fluidity', he pointed out that an increase in temperature served to increase the rate of lipid motion but led to a decrease in lipid order. Since some probes such as the fluorescent probe 1,6-diphenyl-1,3,5-hexatriene (DPH) were more sensitive to lipid order whilst others such as the ESR probe 2N14, were more sensitive to the rate of motion, he suggested that this was likely to be the cause of some of the conflicting results obtained. He also proposed that it would perhaps be more appropriate to consider the effects of lipid composition on hyperthermic cell killing in terms of changes in lipid order due to the inconsistancies he and his coworkers (Lepock et al., 1981; Massicotte-Nolan et al., 1981) had reported when the rate of lipid motion was considered.

In 1983 Lepock et al. (1983) using both the ESR probe 2N14 and the fluorescence polarisation probe DPH conducted a further study for lipid or protein thermotropic transitions in mitochondrial and plasma membranes from Chinese

hamster lung V79 cells near the onset temperatures (40-41.5°C) of hyperthermic cell killing. Whilst they found no lipid transition near 40-41.5°C that could be correlated with hyperthermic killing in either mitochondrial or plasma membranes they did find that measurements of intrinsic protein fluorescence and energy transfer from membrane protein to the lipid-soluble fluorescent probe *trans*-paranaric acid demonstrated the existence of an irreversible transition in protein structure or arrangement above approximately 40°C in both mitochondrial and plasma membranes. Later work by Lepock (Lepock et al., 1988, 1989) has also suggested the importance of thermal denaturation of membrane proteins in hyperthermic killing.

It was therefore of interest to ascertain whether other agents such as local anaesthetics which are known to alter the 'fluidity' of plasma membranes might also affect the thermal sensitivity of the HTC cells in an attempt to establish the significance of membrane fluidity in the hyperthermic response of HTC cells. Such investigations form the basis of work presented in the following chapter.

Chapter V

Effect of Local Anaesthetics on the Thermal Sensitivity, Surface Morphology and Internal Cytoskeleton of HTC Cells

5.1 Introduction

Local anaesthetics have been reported to produce a wide variety of effects on cells. In addition to their anaesthetic action on excitable membranes (Blaustein and Goldman, 1966), they have been shown to have specific effects on membranes. For example, they induce the expansion of membranes (Seeman, 1972), displace Ca^{2+} from membranes (Chen, 1974) and modify the osmotic fragility of erythrocytes (Roth and Seeman, 1971). They also inhibit cell fusion (Poste and Reeve, 1972). Other studies have revealed that local anaesthetics can reversibly disrupt both microtubular and microfilamentous cytoskeletal systems (Nicolson et al., 1976). They also potentiate heat induced inhibition of DNA synthesis (Wong and Dewey, 1983) and loss of DNA polymerase activities (Jorritsma et al., 1984). They have also been proposed to cause an increase in protein mass of the nucleus (Roti-Roti and Wilson, 1984). However, many of these effects are probably secondary to the primary effect of the anaesthetic since, for example, the loss of polymerase activities and increase in protein content of nuclei are only observed in whole cells. There is no effect when isolated polymerase or nuclei are treated with anaesthetic.

Although the molecular events accompanying anaesthetic induced changes are still unclear, the fact that they have been reported to enhance the fluidity of membranes in both natural (Hubbell et al., 1970) and model membranes (Colley and Metcalfe, 1972; Butler et al., 1973) prompted their use in the current study to serve as a further means of investigating the importance of membrane fluidity in the cause of hyperthermic cell death of HTC cells.

As discussed earlier, the idea that membrane fluidity may be important in hyperthermic cell death is not new (see Chapter 4). The evidence from the use

of local anaesthetics for a role of membrane fluidity in cell death is fairly convincing for microorganisms. For example Yatvin (1977) using the local anaesthetic procaine, was able to sensitise *E.coli* to heat, furthermore these workers demonstrated a direct correlation between bacterial hyperthermic survival and anaesthetic potency (Yatvin et al., 1982) which strongly supported the hypothesis that the fluidity of membranes might be a major factor contributing to the death of cells.

Yau (1979) working with murine 3T3 cells and CHO cells also reported a potentiation in hyperthermic killing of cells using the local anaesthetic, procaine. However, although the membrane probe, perylene, indicated a significant increase in fluidity of the anaesthetic treated cell membranes this was not apparent using 1,6,diphenyl-1,-3,5 hexatriene (DPH) as a probe. Konings (1985) showed that the thermosensitivity of both normal mouse fibroblast LM cells and cells that had been modified with respect to the content of polyunsaturated fatty acyl chains of the membrane, could be enhanced by treatment with procaine. LM cells were grown in a serum-free medium supplemented with arachidonic acid (20 : 4) which increased the amount of polyunsaturated fatty acyl chains (PUFA) in the phospholipids from 6.6% to 35.9%. A similar result, again using procaine was reported by Hidvegi et al. (1980) working with ascites tumour cells.

It was therefore of interest to ascertain whether a number of tertiary amine local anaesthetics of increasing potency could be shown to potentiate hyperthermic cell killing in HTC cells in a way that correlated with changes in membrane fluidity, as measured by the steady state fluorescence probe, DPH.

One important factor that needs to be taken into consideration when using tertiary amine local anaesthetics is that they can exist either as positively charged ions (cationic) or as neutral molecules depending on the dissociation constant (pKa) of the anaesthetic and the pH of the buffering medium. For example tetracaine exists purely in the charged cationic form at pH 5.5 and in the neutral form at pH 9.5. A number of studies have shown that the charge of the anaesthetic is important in determining its activity since the charged and uncharged forms have different partition coefficients in the lipid bilayer. Kelusky and Smith (1983) showed that in model phosphatidylcholine (PC) and phos-

phatidylethanolamine (PE) bilayers, the uncharged form is more lipid-soluble due to hydrophobic interactions between the anaesthetic and lipid molecules, whilst for phosphatidylserine, which was negatively charged at the pH studied, the charged form of the anaesthetic was more soluble in the lipid bilayer due to electrostatic interactions between the two opposite charges (Kelusky et al., 1986).

Auger et al. (1988) have shown that the uncharged form of tetracaine can partition deeply into model membranes composed of myristoyl-sn-glycero-3 phosphocholine bilayers whilst the charged form may interact electrostatically with the phospholipid head group and induce a greater fluidity change than the uncharged form.

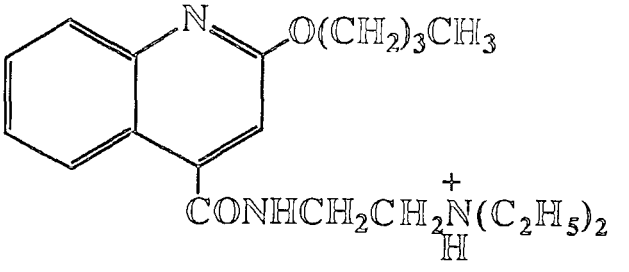
In the current study, to ensure that the anaesthetics dibucaine, tetracaine and procaine were maintained predominantly in their cationic form (figure 5.1), the pH was kept below 7.5 since the pKa values of these anaesthetics are around pH 7.8 (Low et al., 1979). Under these conditions the order of membrane surface adsorption of these cationic forms of local anaesthetic at any given concentration has been shown to be dibucaine > tetracaine > procaine (Ohki, 1984) which reflects the narcotic potencies of these anaesthetics on nerve membrane excitability (Truant and Takman, 1965).

As discussed in Chapter 3, a number of workers have reported morphological changes produced by hyperthermic treatment. Bass et al. (1978) working with CHO cells at $43°C$ noticed that following treatment, the surface was characterised by a reduction in the number of microvilli, a decreased adherence to the substratum and an increase in bleb formation. Similarly a number of studies have shown that hyperthermia causes cells to round up and inhibits their ability to attach to the culture substratum (Lin et al., 1973; Schamhart et al., 1984). Mulcahy et al. (1981) working with P388 ascites tumour cells demonstrated some interesting heat induced morphological differences between cells that had undergone various membrane modifications including treatment with local anaesthetic. Their results were consistent with the hypothesis that a cell's response to hyperthermic insult was related to its membrane fluidity at the time of treatment. It was therefore of interest in the current study to see whether or not the presence

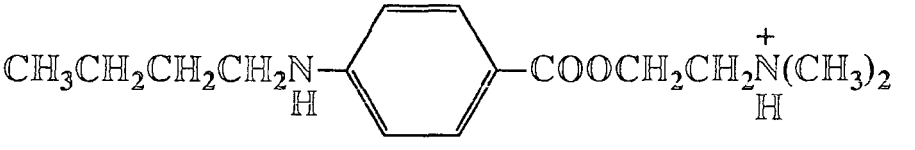
Figure 5.1

Cationic forms of three local anaesthetics used in hyperthermic studies

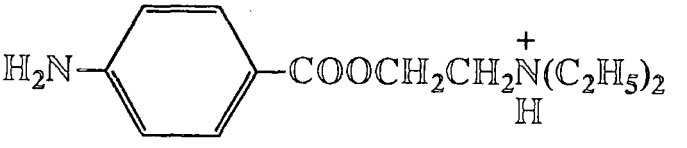
DIBUCAINE



TETRACAINE



PROCAINE



Edstrom et al., 1975; Nicolson et al., 1976) quite probably by an indirect membrane effect, which leads to an intracellular release of calcium (Genna et al., 1980), there are no reports on the effects of anaesthetic either in the presence or absence of heat, on the intermediate filaments of cells. Consequently a study of local anaesthetic effect on the intermediate filament network of heated and control cells was undertaken. Although a number of methods have been used to reveal the intermediate filament network of cells (French et al., 1982; Ishii et al., 1985) the method of Fey et al. (1984) was adopted for two main reasons. Firstly this method avoids the use of conventional embedded sections which can often mask cytoskeletal networks (Fey et al., 1984), secondly it is designed to reveal information on the architecture of the nuclear matrix of a cell as well as on the intermediate filament network which is of interest since the association between intermediate filaments and intact nuclei has frequently been reported (Woodcock, 1980; Capco et al., 1982).

5.2 Materials and Methods

5.2.1 Toxicity Studies

Stock solutions of dibucaine-hydrochloride ($50mM$), tetracaine-hydrochloride ($500mM$) and procaine-hydrochloride ($1000mM$) were prepared in distilled water and filter sterilised. They were stored for a maximum of two weeks at $4°C$.

Ranges of anaesthetic concentrations in normal culture medium were prepared by serial dilution of the above stock solutions with culture medium. Dibucaine was prepared in a final concentration range in the culture medium of $1mM$ to $0.005mM$, tetracaine in the final concentration range $5mM$ to $0.05mM$ and procaine in the highest, final concentration range of $50mM$ to $0.1mM$. Addition of anaesthetic to culture medium did not affect the final pH of the solution.

Cells (5×10^3/well) were seeded into 24-well plates in $1ml$ growth medium on day 0 and incubated at $37°C$ under a humid atmosphere of air/CO_2 ($19:1$, v/v). The medium was changed on day 1. On day 3, the culture medium in wells was replaced with either $1ml$ of culture medium (control cells) or $1ml$ of an anaesthetic in culture medium. In all experiments there was a minimum of 4 wells per experimental condition. The plates were incubated at $37°C$ in an

atmosphere of air/CO_2 (19 : 1, v/v) for a period of 4.5 hours. The medium was then replaced with normal culture medium and the plates returned to the $37°C$ incubator. The next day plates were given another media change. Cell survival was determined on day 6 by the modified Mosmann method described in Chapter 2, Materials and Methods, sectiom 2.2.4(b).

An 'anaesthetic survival value' (ASV) for each concentration of anaesthetic tested was determined as follows:

$$Anaesthetic\ survival\ value = \frac{Incubator\ Anaesthetic\ Absorbance\ value}{Incubator\ control\ Absorbance\ value} \times 100$$

If an anaesthetic has no effect on cell survival $ASV = 100\%$.

5.2.2 Hyperthermic Studies

Concentrations of anaesthetic were selected that decreased the survival of cells incubated at $37°C$ in an atmosphere of air/CO_2, (19 : 1, v/v) by no more than 35% compared to control cells as determined by the toxicity studies.

Cells (5×10^3/well) were seeded into 24-well plates in $1ml$ growth medium on day 0 and incubated at $37°C$ under a humid atmosphere of air/CO_2 (19 : 1, v/v). The culture medium was changed after 24 hours and then on day 3 the medium was replaced with medium that lacked fungizone (Hahn et al., 1977) but contained $10mM$ Hepes, $pH7.4$ (heating medium). This heating medium was prepared with and without the selected concentrations of the various anaesthetics.

Plates were returned to the incubator for 20 minutes to gas. After sealing, plates were equilibrated in a $37°C$ water bath. They were then rapidly transferred to a water bath set at $44.5°C$ ($\pm 0.1°C$) for 5 minutes before final transfer to a water bath at the desired hyperthermic temperature of $43°C$ ($\pm 0.1°$). The lag time for thermal equilibration, which was considerably reduced by this protocol, was included in the overall heating time. The medium was replaced with normal culture medium in each plate immediately after heating and the plates were returned to the $37°C$ incubator.

A further media change was made the next day and the plates were then assayed for cell survival on day 6 by the modified Mosmann method described in

Chapter 2, Materials and Methods, section 2.2.4(b). Cell survival, after anaesthetic treatment, was defined as the survival measured in the presence of anaesthetic at $43°C$ relative to that measured in the presence of the same anaesthetic at $37°C$ in the $37°C$ water bath which was taken as 100%. Control cell survival at $43°C$ was similarly defined as the survival of control cells at $43°C$ relative to the survival of control cells at $37°C$ in the $37°C$ bath.

Anaesthetic survival values were also determined from control plates kept in the $37°C$ incubator for the duration of the experiment.

5.2.3 *pH* Determinations

Three batches of heating medium, containing the highest final concentration of anaesthetic used in heating experiments ($0.05mM$ dibucaine, $0.2mM$ tetracaine or $5mM$ procaine) were prepared by serial dilution of the stock anaesthetic solutions in heating medium.

Plates containing heating medium alone or heating medium containing the different concentrations of anaesthetic were either placed in the incubator at $37°C$ for 3.5 hours or heated, after gassing, at $37°C$ and $43°C$, as described above, for varying periods up to 3.5 hours. The *pH* of the various types of media was measured at appropriate time points using a Phillip Harris digital *pH* meter.

5.2.4 D.P.H. Steady State Fluorescence Polarisation

HTC cell plasma membranes were isolated by method 2, outlined in Chapter 4, Materials and Methods, section 4.2.1(c)ii. The membranes were prepared for fluorescence polarisation measurements as described in Chapter 4, Materials and Methods, section 4.2.3.

Two types of study were performed.

(i) Changes in polarisation with temperature

A concentrated solution of dibucaine was added to a cuvette containing membranes to give a final concentration of $0.05mM$, the same as that used in the hyperthermic studies. A similar cuvette was prepared in the absence of dibucaine. The cuvettes were left to stand for 15 minutes at room temperature to

enable the reagent to equilibrate with the membranes. The change in polarisation against temperature over the temperature range 4° to 50°C was then carried out as described earlier in Chapter 4, Materials and Methods, section 4.2.3.

(ii) Changes in polarisation with anaesthetic concentration

The fluorescence polarisation of membranes present in three separate cuvettes was measured at 37°C. Then concentrated solutions of dibucaine, tetracaine and procaine were prepared such that when $10\mu l, 20\mu l$ or $25\mu l$ volumes respectively were added to the membranes present in the 3 cuvettes they gave final anaesthetic concentrations of $0.05mM$ dibucaine, $0.2mM$ tetracaine and $1.0mM$ procaine, reflecting the final concentrations of anaesthetics used in the hyperthermic studies.

After leaving the cuvettes to stand for 15 minutes at room temperature, so that the reagent could equilibrate with the membranes, the fluorescence polarisation values of the 3 cuvettes were measured again at 37°C. A further addition of each concentrated anaesthetic solution was then made to the appropriate cuvette, effectively doubling the final concentration of anaesthetic present and a further polarisation measurement was determined at 37°C. Five sequential additions of the anaesthetics were made to determine the effect of the concentration of the anaesthetic on the fluidity change.

5.2.5 Surface Morphology Studies

Cells (3.5×10^4/well) were seeded into 24-well plates containing coverslips on day 0 in $1ml$ growth medium and incubated at 37°C under a humid atmosphere of air/CO_2 (19 : 1, v/v). The medium was changed on day 1. On day 2 cells were exposed either to normal heating medium or to heating medium containing $0.20mM$ dibucaine (ASV = 75.3%). The cells were then gassed for 20 minutes in the 37°C incubator. After gassing the cells were either maintained in the incubator for a further one hour period, or sealed and heated at 43°C for 1 hour. Following treatment, all cells were washed once with $1ml$ of calcium and magnesium-free phosphate buffered saline (Ca^{2+}-, Mg^{2+}-free PBS) and then fixed on the coverslips in $1ml$ of Karnovsky fixative, post-fixed in osmium tetroxide (1% w/v) in sodium cacodylate ($0.1M$), dehydrated through an

ethanol series, dried through the CO_2 critical point and sputter coated with gold-palladium by the methodology described in Chapter 2, Materials and Methods, section 2.2.2(b), for examination in a Stereoscan 800 scanning electron microscope.

5.2.6 Nuclear Matrix - Intermediate Filament (NM-IF) Studies

The detergent extraction method of Fey et al. (1984), originally developed to study the nuclear matrix-intermediate filament arrangement in colonies of Madin-Darby canine kidney cells, was adopted in the current investigations, with the modifications given in their later paper (Fey and Penman, 1984).

HTC cells were grown on either glass coverslips or on formvar and carbon coated gold grids in 24 well plates. Wells seeded with 3.5×10^4 cells on day 0 were treated with or without $0.20mM$ dibucaine in the presence of absence of heat, as described in section 5.2.5 above.

After treatment the grids and coverslips were rinsed twice with Ca^{2+}-, Mg^{2+}-free PBS, taking care not to direct the Ca^{2+}-, Mg^{2+}-free PBS onto the surface of the grids and coverslips and so dislodge cells. The grids and coverslips were then extracted with cytoskeleton (CSK) buffer ($100mM$ $NaCl$, $300mM$ sucrose, $10mM$ Piperazine-NN'-bis-2-ethanesulphonic acid (PIPES) (pH 6.8), $3mM$ $MgCl_2$, 0.5% (w/v) Triton X-100 and $1.2mM$ phenylmethylsulphonylfluoride) for 10 minutes at $0°C$ (Extraction 1) and the resulting soluble fraction was removed.

Various types of cells including HeLa, 3T3 and CHO cell lines have been reported to retain their surface morphology after extraction with Triton X-100 in CSK buffer. To examine whether this was also the case with HTC cells, coverslips bearing Triton X-100 extracted cells from this first extraction step were fixed in CSK buffer containing 2.5% (w/v) glutaraldehyde for 30 minutes at $0°C$. The extracted cells on the coverslips were then rinsed in $0.1M$ sodium cacodylate buffer pH 7.3 and post-fixed in 1% (w/v) osmium tetroxide in $0.1M$ sodium cacodylate buffer, pH 7.3, for 0.5-1 hour at $4°C$. Following post-fixation the cells on the coverslips were dehydrated through an ethanol series, dried through the

CO_2 critical point and sputter coated with gold-palladium as described in Chapter 2, Materials and Methods, section 2.2.2(b), for examination in the Stereoscan 800 scanning electron microscope.

The cytoskeleton fraction from cells grown on gold grids was removed by subjecting the Triton X-100 insoluble structures remaining from the first extraction step, to a second extraction buffer ($250mM$ ammonium sulphate, $300mM$ sucrose, $10mM$ PIPES (pH 6.8), $3mM$ $MgCl_2$, $1.2mM$ phenylmethylsulphonylfluoride and 0.5% (w/v) Triton X-100) for 10 minutes at $0°C$ (Extraction 2).

Finally, to remove the chromatin fraction from the HTC cells the material that was left from the two earlier extraction steps was exposed to a buffer identical to CSK except that it contained $50mM$ $NaCl$ as opposed to $100mM$ $NaCl$, and in addition it also contained $400\mu g/ml$ pancreatic DNase and $400\mu g/ml$ pancreatic RNase. Digestion was permitted to proceed for 20 minutes at $20°C$ before ammonium sulphate was added, to a final concentration of $0.25M$, to elute the chromatin associated proteins during a further 5 minute incubation period also at $20°C$ (Extraction 3).

Grids bearing the resultant salt-resistant NM-IF structure were fixed in the final extraction buffer containing $50mM$ $NaCl$ as opposed to $100mM$ $NaCl$, in the presence of 2.5% (w/v) glutaraldehyde but in the absence of the DNase and RNase enzymes and ammonium sulphate for 30 minutes at $0°C$. The material on the grids was then rinsed in $0.1M$ sodium cacodylate buffer, pH 7.3, for 0.5-1 hour at $4°C$. Once again, following post-fixation the material on the grids was dehydrated through an ethanol series, dried through the CO_2 critical point and sputter coated with gold-palladium by the methodology described in Chapter 2, Materials and Methods, section 2.2.2(b). Grids were then examined as whole mounts in a Phillips 400T transmission electron microscope.

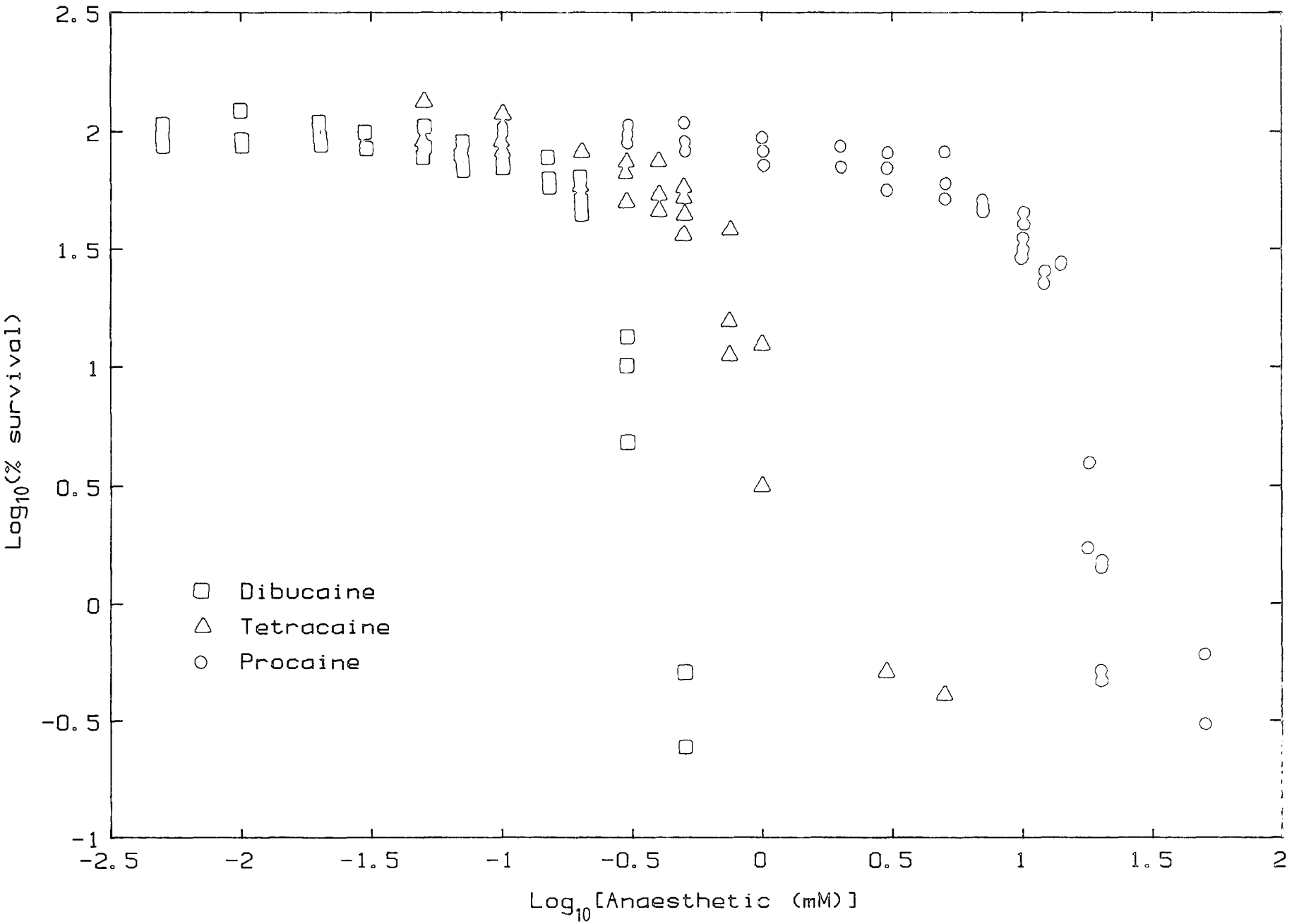
5.3 Results

Figure 5.2 shows the effect of increasing concentrations of the three local anaesthetics dibucaine, tetracaine and procaine on cell survival at $37°C$. It can be seen that there is considerable variation in the potency of these drugs with dibucaine being the most potent, followed by tetracaine then procaine. When

Figure 5.2

The effect of anaesthetic concentration on cell survival at 37°C

Plates (24-well) were seeded with 5×10^3 cells/well on day 0 and maintained in an incubator at $37°C$ under a humid atmosphere of air/CO_2 (19 : 1, v/v) at all times. The medium was changed on day 1. On day 3 cells were exposed to a range of dibucaine, procaine and tetracaine concentrations for a period of $4\frac{1}{2}$ hours. Cells were returned to normal medium after exposure. The medium was changed on day 4. Cell survival was determined on day 6 by the modified Mosmann method described in Chapter 2, Materials and Methods, section 2.2.4(b).


2.5
2
1.5
1
0.5
0
-0.5
-1
-2.5
-2
-1.5
-1
-0.5
0
0.5
1
1.5
2
Log10(% survival)
Log10[Anaesthetic (mM)]
Dibucaine
Tetracaine
Procaine

wells are inoculated with 5×10^3 cells, allowed to grow for three days and are then exposed to the stated concentrations of anaesthetic, it can be seen that to reduce cell survival by 50% requires only a $0.2mM$ final concentration of dibucaine, a $0.4mM$ final concentration of tetracaine but a $6mM$ final concentration of procaine. The curves obtained with the three anaesthetics are very similar and indicate that at low anaesthetic concentrations there is very little effect on cell survival. However, as the concentration of anaesthetic is increased it appears to reach a critical level above which dramatic reductions in cell survival result.

Concentrations of the three anaesthetics were selected that were below this 'critical' concentration and produced a decrease in cell survival at $37°C$ of no more than 35%.

Figures 5.3, 5.4 and 5.5 show the effect of the presence of various concentrations of anaesthetic on the response of HTC cells to heat at $43°C$ followed over a 3.5 hour period. It can be seen that as the concentration of anaesthetic in the heating medium increases there is a corresponding decrease in cell survival. Closer examination of figures 5.3, 5.4 and 5.5 reveal that following longer periods of incubation at $43°C$ in the absence of anaesthetics or in the presence of low concentrations of anaesthetics, there is a slight decline in the rate of hyperthermic cell death as measured by the colorimetric assay. As the concentration of the anaesthetics is increased this non-linearity disappears.

As discussed in Chapter 3, the non-linear/biphasic nature of this assay which is apparent, is thought to reflect differences in the nature of the cell death that occurs after hyperthermic treatment (Vidair and Dewey, 1988). It has been proposed that cells may die quickly following heat treatment (rapid cell death) or after a much longer time period (slow cell death). Since this assay is performed 3 days post heating, whilst it is likely to measure any rapid cell death that has occurred it is unlikely to monitor the slow cell death since this has been shown to take up to a week post heating to become evident (Vidair and Dewey, 1988). Hence the plateau regions that are seen in figures 5.3, 5.4 and 5.5 would appear to result from the presence of cells affected by the slow mode of cell death which will be lost from the culture surface with time but which at the period of assay are still metabolically active and therefore recorded as cells which have survived

Figure 5.3

The effect of dibucaine on cell survival at 43°C

Plates (24-well) were seeded with 5×10^3 cells/well on day 0 and maintained in an incubator at $37°C$ under a humid atmosphere of air/CO_2 (19 : 1, v/v). The medium was changed on day 1. On day 3 cells were exposed to $0.05mM$ dibucaine for periods up to $3\frac{1}{2}$ hours at $43°C$. After heating, the medium was replaced with normal medium and the cells were returned to the $37°C$ incubator. The medium was changed on day 4 and cell survival was determined on day 6 by the modified Mosmann method described in Chapter 2, Materials and Methods, section 2.2.4(b).

The figure shows data derived from two separate experiments. Best fit regression lines have been fitted to the data using the straight line equation $y = a + bx$.

Correlation coefficients of regression lines:

Control -0.953

$0.05mM$ Dibucaine -0.967

Anaesthetic survival value:

$0.05mM$ Dibucaine = 84.5%

The anaesthetic survival value was determined as described by Materials and Methods, section 5.2.1, and represents the average value obtained from the two experiments.

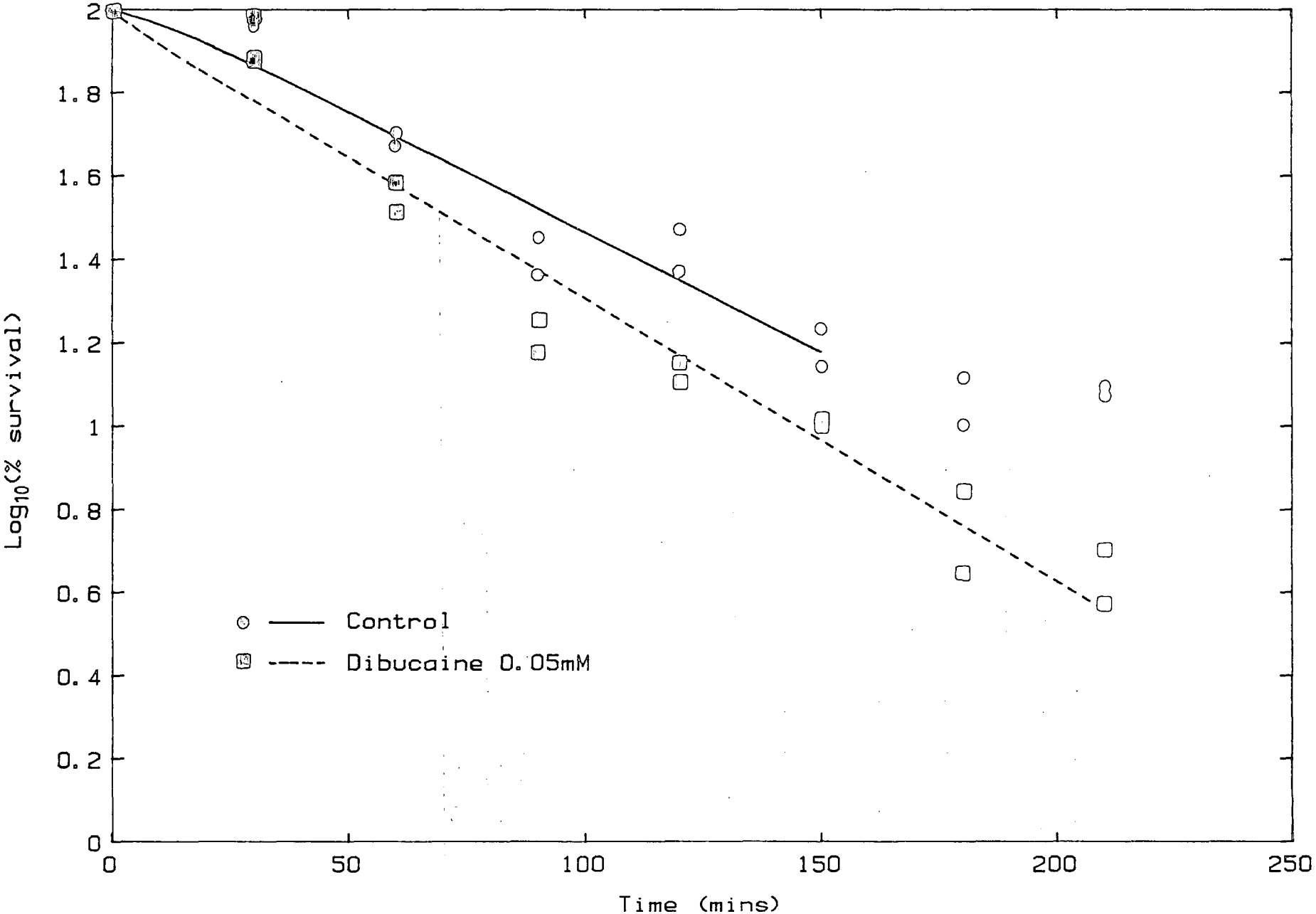

2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Log10(% survival)
0
50
100
150
200
250
Time (mins)
Control
Dibucaine 0.05mM

Figure 5.4

The effect of tetracaine on cell survival at 43°C

Plates (24-well) were seeded with 5×10^3 cells/well on day 0 and treated as described in the legend of figure 5.3 but using $0.05mM$, $0.1mM$ and $0.2mM$ tetracaine.

The figure shows data derived from up to three separate experiments. Best fit regression lines have been fitted to the data using the straight line equation $y = a + bx$.

Correlation coefficients of regression lines:

Control	-0.963
$0.05mM$ Tetracaine	-0.984
$0.1mM$ Tetracaine	-0.989
$0.2mM$ Tetracaine	-0.941

Anaesthetic survival values:

$0.05mM$ Tetracaine $= 91.4\%$

$0.1mM$ Tetracaine $= 86.7\%$

$0.2mM$ Tetracaine $= 79.5\%$

The anaesthetic survival value was determined as described by Materials and Methods, section 5.2.1, and represents the average value obtained from the two experiments.

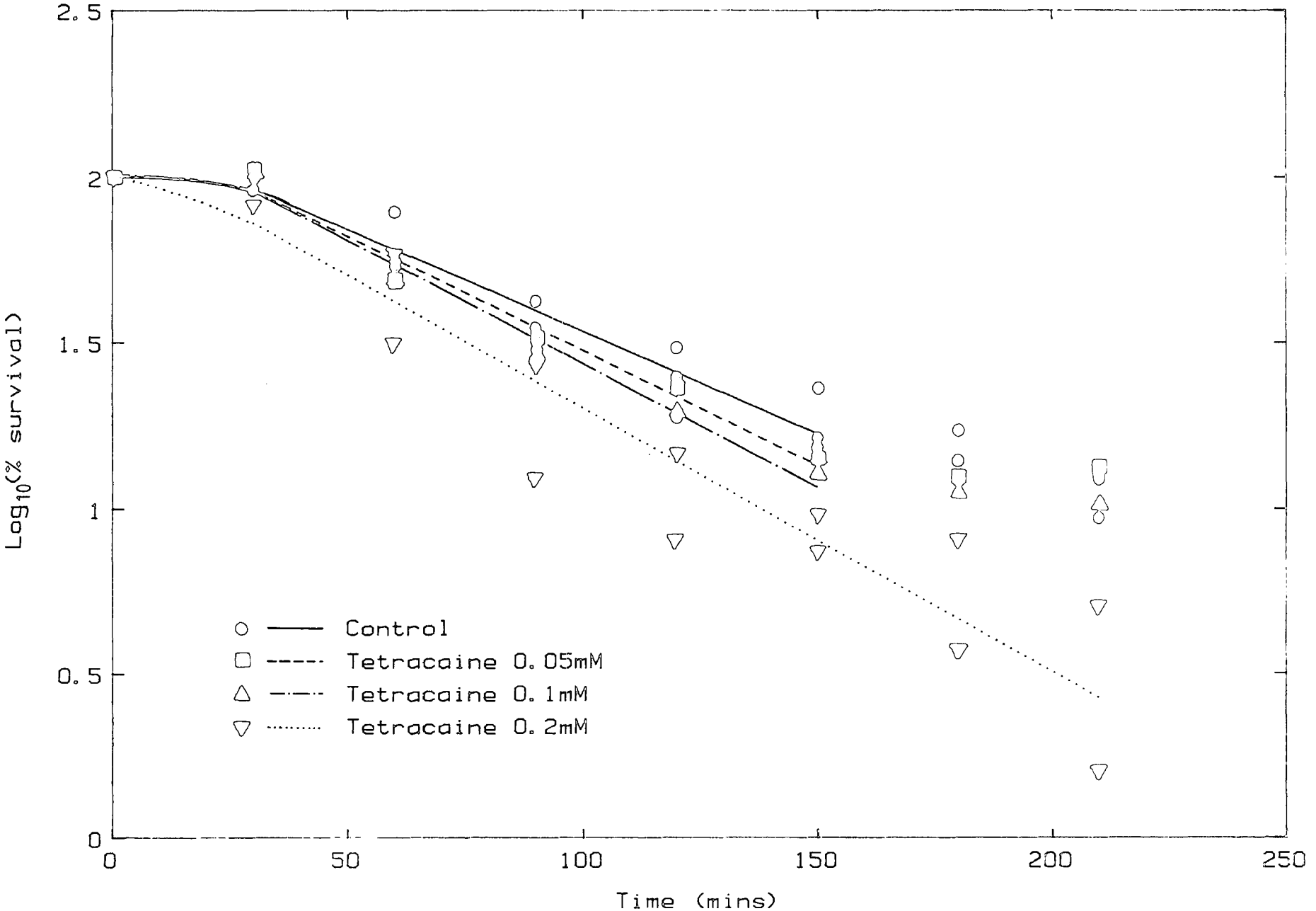

2.5
2
1.5
1
0.5
0
0
50
100
150
200
250
Log10(% survival)
Time (mins)
Control
Tetracaine 0.05mM
Tetracaine 0.1mM
Tetracaine 0.2mM

Figure 5.5

The effect of procaine on cell survival at 43°C

Plates (24-well) were seeded with 5×10^3 cells/well on day 0 and treated as described in the legend of figure 5.3 but using $1mM$ and $5mM$ procaine.

The figure shows data derived from up to four separate experiments. Best fit regression lines have been fitted to the data using the straight line equation $y = a + bx$.

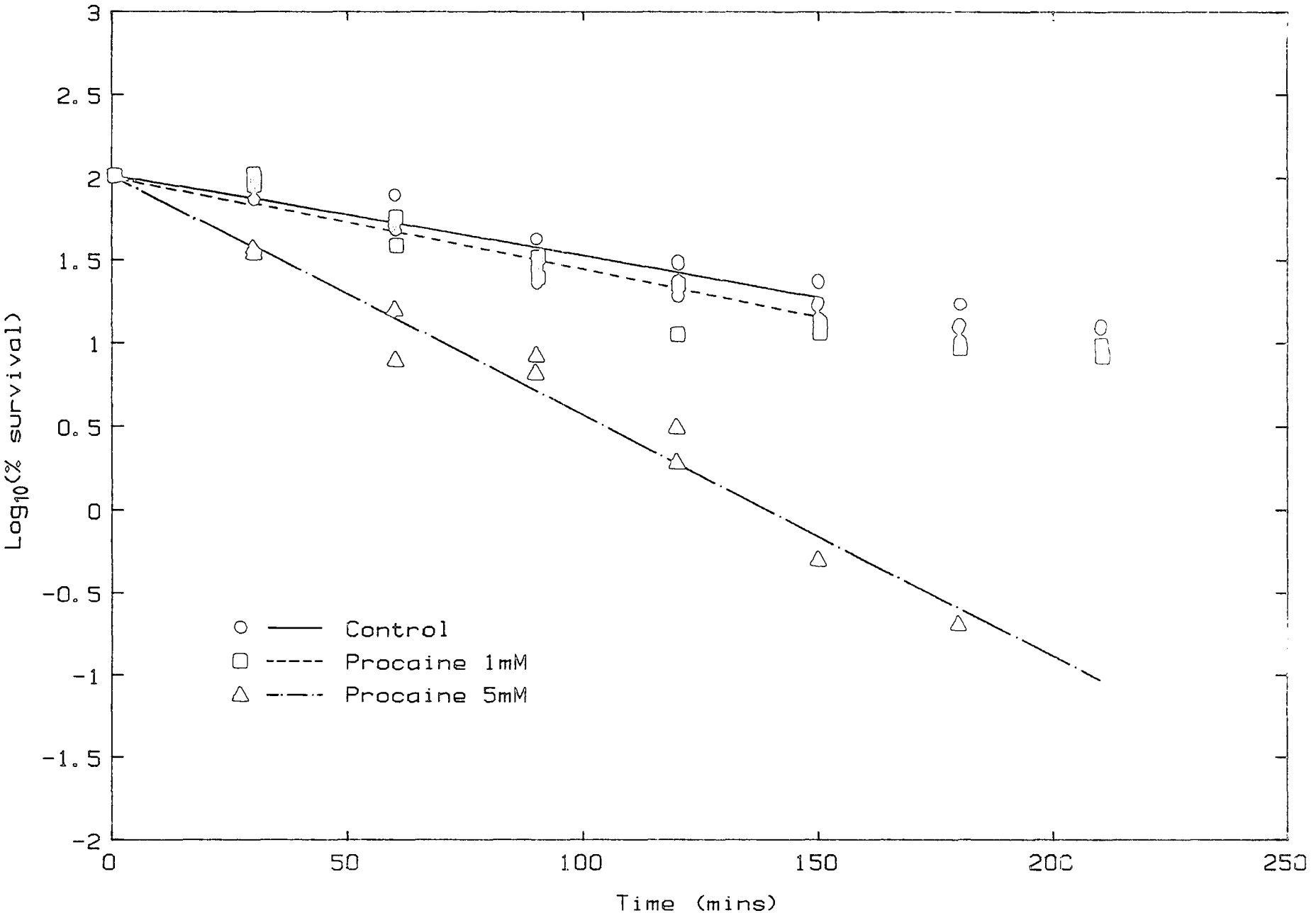
Correlation coefficients of regression lines:

Control	−0.940
$1mM$ Procaine	−0.956
$5mM$ Procaine	−0.979

Anaesthetic survival values:

$1mM$ Procaine	= 84.3%
$5mM$ Procaine	= 70.7%

The anaesthetic survival value was determined as described by Materials and Methods, section 5.2.1, and represents the average value obtained from the two experiments.


3
2.5
2
1.5
1
0.5
0
-0.5
-1
-1.5
-2
0
50
100
150
200
250
Log10(% survival)
Time (mins)
Control
Procaine 1mM
Procaine 5mM

the hyperthermic insult. Increasing the concentration of the anaesthetics would appear to produce a similar result as an increase in temperature (compare figures 5.4 and 5.5 with figure 3.8) since both conditions produce linear survival curves, suggesting an increase in cell killing by the rapid mode of death.

For statistical analysis, linear regression lines have been fitted to the initial linear regions of the biphasic survival curves and the times required to kill 25%, 50% and 75% of the cells (LD_{25}, LD_{50} and LD_{75}) have been used as an index of cytotoxicity since these parameters occur in the regions of the curves where cell death is most likely to be caused by the rapid mode.

Table 5.1 shows the time taken to achieve LD_{25}, LD_{50} and LD_{75} in the presence and absence of anaesthetics. It can be seen that apart from $5mM$ procaine, the effect of the anaesthetics at the LD_{25} level is not substantial, i.e. the LD_{25} value obtained with control cells is not reduced appreciably by the presence of anaesthetics. However, whilst none of the LD_{25} values are significantly different from control LD_{25} values at the 95% confidence level ($p = 0.05$), it is obvious that in the presence of $5mM$ procaine there is a marked reduction in hyperthermic cell survival at the LD_{25} level. At the LD_{50} level it can be seen that although the presence of anaesthetic reduces the time taken to kill 50% of cells present, it is only the result obtained in the presence of $5mM$ procaine that is significant ($p < 0.01$). $5mM$ procaine would appear to reduce the LD_{50} by 45 minutes. At the LD_{75} value the higher concentrations of all anaesthetics used produced a significant reduction in the LD_{75} value at the 95% confidence level. In fact, the result obtained with $5mM$ procaine is significant at the 99.9% level of confidence ($p = 0.001$). Dibucaine, at a concentration of $0.05mM$, would appear to reduce the LD_{75} figure by 25 minutes, $0.2mM$ tetracaine would appear to reduce the LD_{75} figure by 33 minutes whilst $5mM$ procaine produces the greatest effect, reducing the LD_{75} value by 75 minutes.

Table 5.2 shows the variation in pH of heating medium and medium containing anaesthetic that occurred over a 3.5 hour period at $43°C$. It can be seen that pH does not alter significantly during the heating period although all values show a slight increase when compared to medium maintained in the incubator at $37°C$ in an atmosphere of air/CO_2 ($19:1$, v/v).

Table 5.1

The Influence of anaesthetics on LD_{25}, LD_{50} and LD_{75} values (mins) for HTC cells heated at 43°C

Anaesthetic (mM)	LD_{25}	LD_{50}	LD_{75}
Dibucaine			
0	28.6 ± 8.4 (10)	59.2 ± 5.9 (10)	111.6 ± 5.3 (10)
0.05	16.1 ± 9.1 (14)	42.0 ± 7.4 (14)	86.3 ± 5.2 (14)†
Tetracaine			
0	44.8 ± 4.8 (15)	72.8 ± 3.5 (15)	120.7 ± 4.0 (15)
0.05	42.0 ± 6.6 (5)	67.5 ± 5.0 (5)	112.0 ± 4.9 (5)
0.10	40.9 ± 5.5 (5)	64.6 ± 4.2 (5)	105.1 ± 3.8 (5)
0.20	28.1 ± 11.4 (14)	50.2 ± 9.6 (14)	88.0 ± 7.1 (14)†
Procaine			
0	37.2 ± 5.8 (20)	66.8 ± 4.1 (20)	117.6 ± 4.3 (20)
1	36.6 ± 7.4 (10)	59.9 ± 5.7 (10)	99.8 ± 4.7 (10)
5	9.9 ± 7.0 (10)	22.0 ± 6.3 (10)‡	42.8 ± 5.1 (10)*

The errors shown are derived from statistical analysis of regression lines using Student's t-distribution following the method of Dr. Seheult.

Numbers in brackets represent number of observations

† significantly different from control value at $p = 0.05$

‡ significantly different from control value at $p = 0.01$

* significantly different from control value at $p = 0.001$

Table 5.2

The Influence of temperature on pH of medium in wells in the absence and presence of anaesthetics

Heating medium was prepared in the absence or presence of the stated concentrations of anaesthetics and added to wells of six 24-well plates. After gassing for 20 minutes in the $37°C$ incubator in an atmosphere of air/ CO_2 ($19:1$, v/v), one plate was maintained in the incubator, one plate was maintained at $37°C$ in a water bath and the remaining four plates were heated at $43°C$ in a water bath. pH determinations were made at the time points given below. The results represent data from a single experiment.

Temperature ($°C$)	Time (mins)	pH value			
		Normal	Dibucaine $0.05mM$	Tetracaine $0.2mM$	Procaine $5mM$
37 Incubator	210	7.41	7.43	7.43	7.41
37 Bath	210	7.61	7.59	7.60	7.58
43 Bath	30	7.61	7.60	7.63	7.61
43 Bath	90	7.62	7.63	7.71	7.64
43 Bath	150	7.59	7.68	7.64	7.61
43 Bath	210	7.75	7.64	7.61	7.66

Figure 5.6 shows the effect of dibucaine at the $0.05mM$ concentration used in hyperthermic studies, on the steady state polarisation of DPH in purified HTC cell plasma membranes in the temperature range 4-50°C. The plot is derived from a single membrane isolation and the equations of the two lines are given in the legend of figure 5.6.

As discussed in Chapter 4, fluorescence polarisation measurements provide information on the physical state of the fatty acyl chains of the phospholipids comprising the membrane bilayer structure, (Stubbs and Smith, 1984) in terms of their angular range of motion (degree of order). This polarisation measurement is often used as an indication of membrane 'fluidity'. The greater the polarisation value obtained, the more 'ordered' and hence less 'fluid' the membrane is considered to be.

In figure 5.6 the large difference in the values of the intercepts indicates a decrease in membrane order brought about by the presence of the anaesthetic. Dibucaine displaces the polarisation measurements by 6°C along the temperature axis. The fact that the gradients of the slopes are not substantially different suggests that the effect of dibucaine on membrane order does not alter across the range of temperatures measured. Further preparations would be required to confirm the significance of this data.

Figure 5.7 shows the effect of varying dibucaine, tetracaine and procaine concentrations on the steady state polarisation of DPH in purified HTC cell plasma membranes at 37°C. Increasing the concentration of dibucaine produces a corresponding decrease in membrane order indicated by a decrease in the polarisation values obtained. The concentrations of dibucaine used in this study on membrane 'fluidity' ranged from $0.05mM$ which was the concentration adopted in the hyperthermic studies, up to $0.2mM$. The fact that $0.05mM$ dibucaine did not affect cell survival at 37°C (figure 5.2) but did produce a decrease in membrane order (figure 5.6) and a decrease in cell survival at 43°C (figure 5.3) suggests the importance of the increased membrane fluidity of the membranes in the killing process.

Similarly, from figure 5.7 it is evident that increasing concentrations of tetracaine produce a corresponding decrease in polarisation values at 37°C suggesting

Figure 5.6

The effect of dibucaine on the steady state fluorescence polarisation of D.P.H. in HTC cell plasma membranes

The figure shows the effect of $0.05mM$ dibucaine on the polarisation of D.P.H. in the temperature range 4-50°C. The plot is derived from a single membrane preparation.

Best fit regression lines were fitted to the data using the straight line equation $y = a + bx$. Values of a (intercept on y axis) and b (gradient) are given below.

Dibucaine concentration (mM)	a	b ($\times 10^2$)
0	0.424	−0.373
0.05	0.403	−0.379

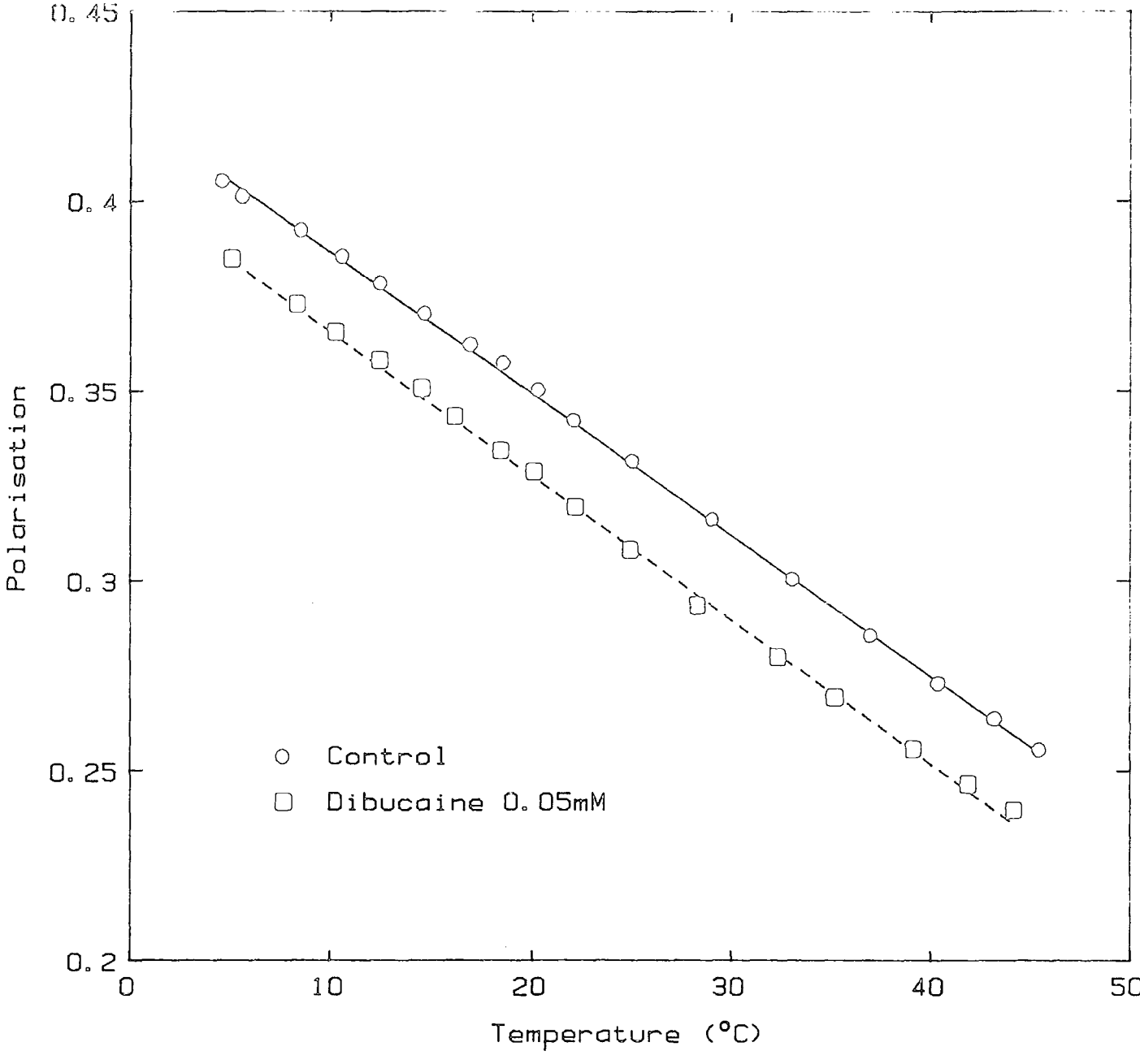
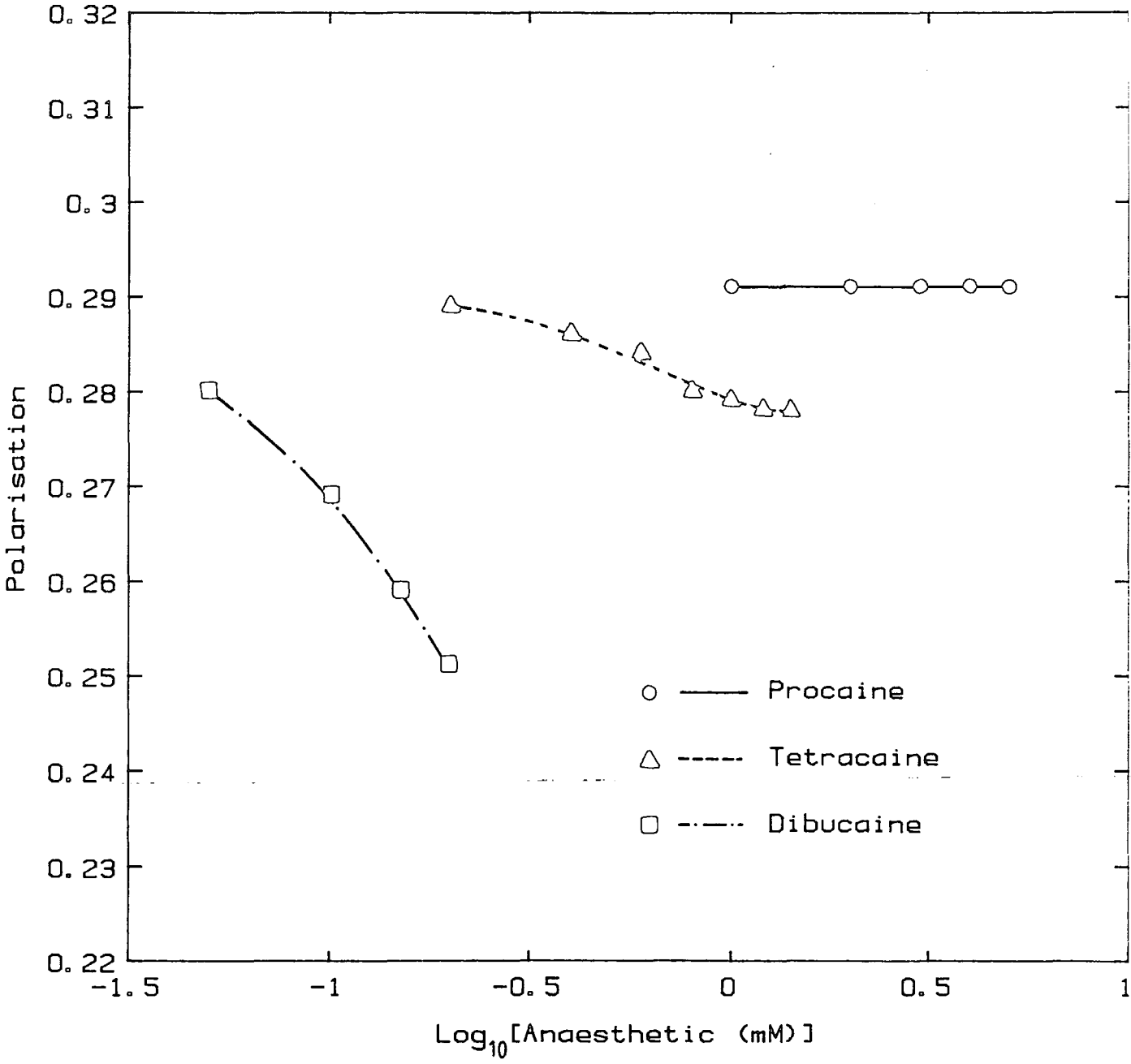

0.45
0.4
0.35
0.3
0.25
0.2
Polarisation
0
10
20
30
40
50
Temperature (°C)
Control
Dibucaine 0.05mM

Figure 5.7

The effect of increasing concentrations of dibucaine, tetracaine and procaine on the steady state fluorescence polarisation of D.P.H. in HTC cell plasma membranes at $37°C$

The figure shows the effect of dibucaine ($0.05mM$ to $0.2mM$), tetracaine ($0.2mM$ to $1.4mM$) and procaine ($1mM$ to $5mM$) on the polarisation of D.P.H. at $37°C$. The plots are derived from a single membrane preparation.

The polarisation values measured for membranes at $37°C$ in the absence of dibucaine, tetracaine and procaine were 0.296, 0.293 and 0.291 respectively.


0.32
0.31
0.3
0.29
0.28
0.27
0.26
0.25
0.24
0.23
0.22
Polarisation
-1.5
-1
-0.5
0
0.5
1
Log10[Anaesthetic (mM)]
Procaine
Tetracaine
Dibucaine

a decrease in membrane order.

The results obtained with procaine however, are difficult to explain since increasing the concentration of this anaesthetic from $1mM$ to $5mM$ had no effect on the steady state polarisation values obtained at $37°C$, and yet $5mM$ procaine, which did not affect cell survival at $37°C$ (figure 5.2), produced a highly significant decrease in cell survival at $43°C$ (figure 5.5). This result would suggest that procaine serves to increase hyperthermic cell death by a different mechanism to the remaining anaesthetics. It could be that this anaesthetic exerts an effect without partitioning significantly into the membrane, i.e. it has a surface effect. Further preparations would be required to confirm the significance of this data.

Figure 5.8 shows the effect of the presence or absence of heat on surface morphology of control and dibucaine treated HTC cells. A concentration of dibucaine was used in this study which reduced cell survival at $37°C$ by no more than 24.7% (i.e. $A.S.V. = 75.3\%$).

Control cells growing at $37°C$, can be seen to form fairly flat colonies of cells which spread out over the available substratum (figure 5.8(a)). The surface of the cells are characterised by numerous microvilli. When anaesthetic is added to the medium the cells adopt a more rounded morphology (figure 5.8(b)) but the cell surface is still characterised by the presence of microvilli.

When control cells are heated at $43°C$ for 1 hour, they assume a more rounded morphology (figure 5.8(c)) but are still characterised by a large number of microvilli. However cells which have been treated with dibucaine and heated at $43°C$ for 1 hour show a striking change in morphology (figure 5.8(d)). Microvilli are lost from the cell surface and the cell surface itself is characterised by a high degree of deformation in the form of pits in the cell surface.

Figures 5.9 and 5.10 show the results of the NM-IF studies performed with HTC cells that were either maintained at $37°C$ for 1 hour in the presence or absence of $0.2mM$ dibucaine or heated at $43°C$ for 1 hour again in the presence or absence of $0.2mM$ dibucaine.

The two scanning electron micrographs in figure 5.9 show the surface morphology of control HTC cells, at $37°C$, before (figure 5.9(a)) and after (figure

Figure 5.8

Scanning electron micrographs showing the effect of heat and/or dibucaine on the surface morphology of HTC cells

HTC cells grown on glass coverslips were treated, fixed, dehydrated, dried through the CO_2 critical point and sputter coated with gold palladium as described in Materials and Methods, section 5.2.5.

(a) Control HTC cells. The bar represents $20\mu m$.

(b) HTC cells treated with $0.2mM$ dibucaine for 1 hour. The bar represents $10\mu m$.

(c) Control HTC cell heated for 1 hour at $43°C$. The bar represents $4\mu m$.

(d) HTC cells treated with $0.2mM$ dibucaine and heated for 1 hour at $43°C$. The bar represents $4\mu m$.

a

b

c

d

Figure 5.9

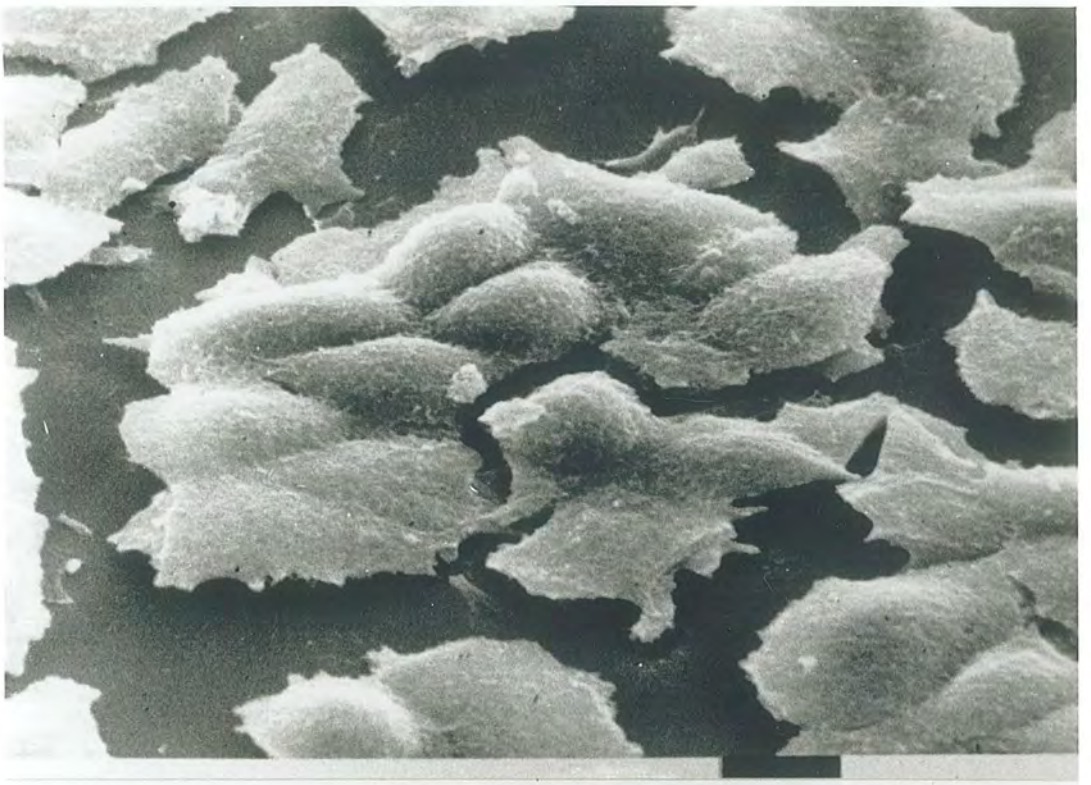
Scanning electron micrographs of HTC cell surface morphology

HTC cells grown on glass coverslips were treated, fixed, dehydrated, dried through the CO_2 critical point and sputter coated with gold palladium as described in Materials and Methods, section 5.2.6.

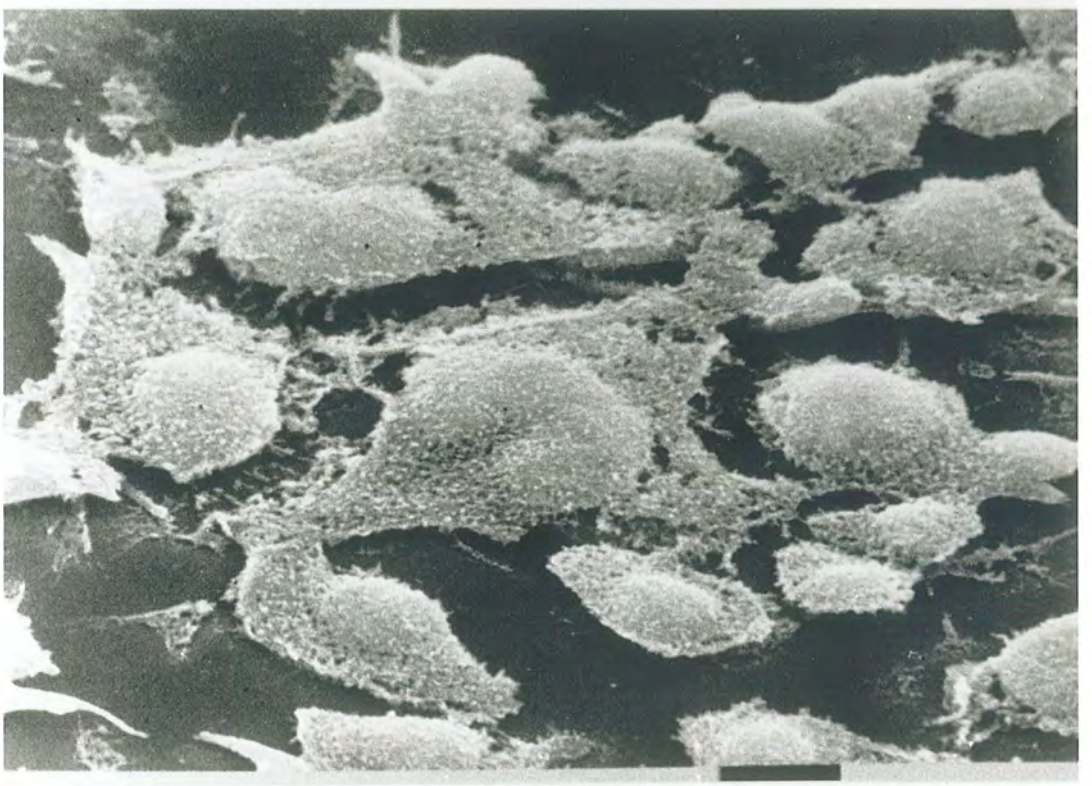
(a) Whole cell surface morphology.

(b) Cell surface morphology after extraction in 0.5% Triton X-100 (Extraction 1).

The bar in each figure represents $10\mu m$.



a



b

Figure 5.10

Whole mount Transmission electron micrographs of the NM-IF scaffold in HTC cells

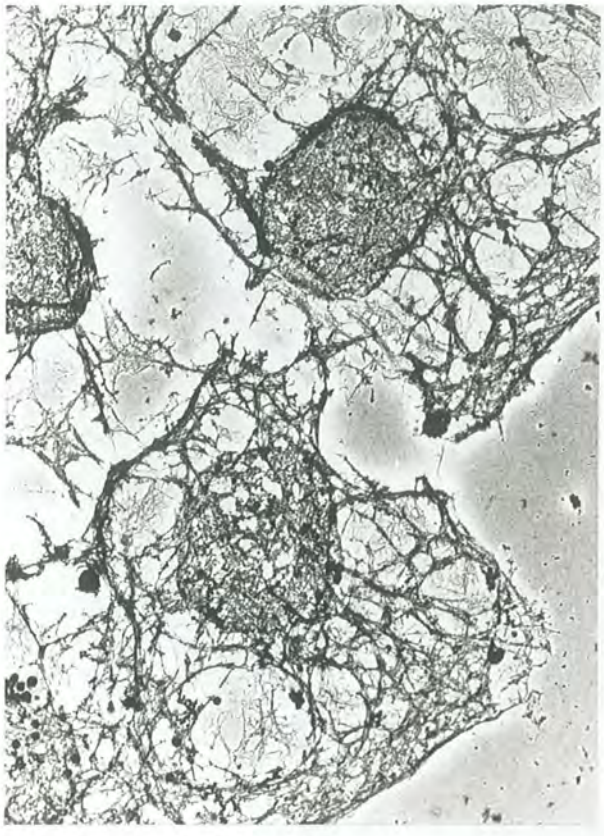
HTC cells grown on gold grids were treated, fixed, dehydrated, dried through the CO_2 critical point and sputter coated with gold palladium as described in Materials and Methods, section 5.2.6.

(a) NM-IF scaffold in control HTC cells (×2200).

(b) NM-IF scaffold in HTC cells treated with $0.2mM$ dibucaine for 1 hour (×2900).

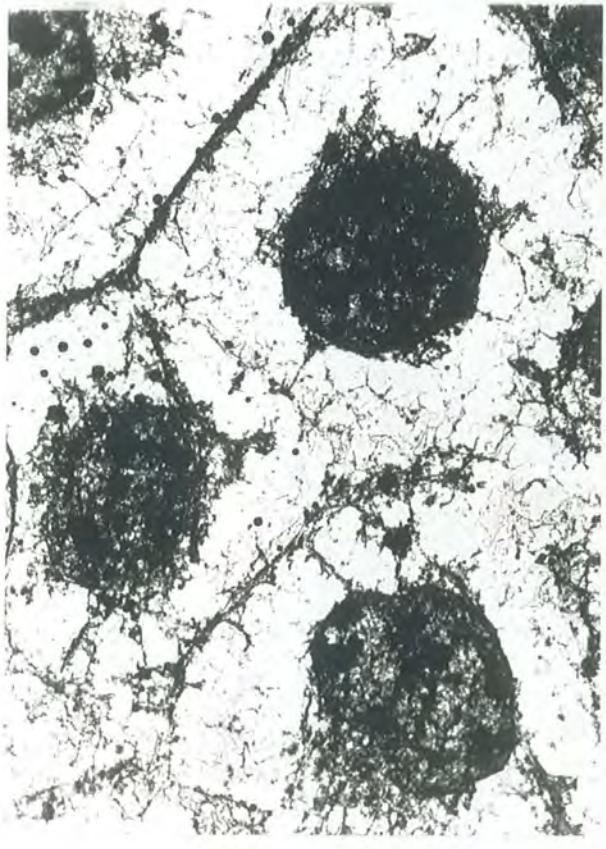
(c) NM-IF scaffold in control HTC cells heated for 1 hour at $43°C$ (×2200).

(d) NM-IF scaffold in HTC cells treated with $0.2mM$ dibucaine and heated for 1 hour at $43°C$ (×2200).

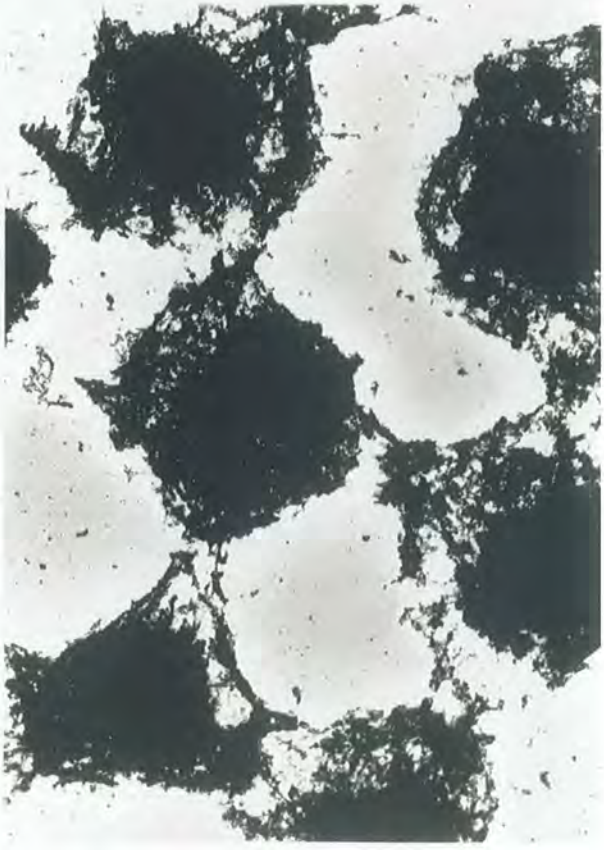


a

b



c



d

5.9(b)) extraction 1. It is evident that after the first extraction process, which is thought to remove soluble proteins and most of the lipids (Fey et al., 1984), the configuration of the cellular surface remains essentially unchanged suggesting the existence of some form of supportive internal skeletal framework.

Figure 5.10 shows a series of whole mount transmission electron micrographs of HTC cells that have undergone the three extraction steps of the NM-IF preparation procedure. Because of the rounding up process that tends to occur during heating which is enhanced when anaesthetic is present, the extraction process proved difficult with the heat treated cells, and the attempts at chromatin removal to expose the nuclear matrix architecture were not successful. However, in terms of the intermediate filament arrangement of the cells under the various conditions of treatment it can be seen that whilst the control cell cytoplasm (figure 5.10(a)) is characterised by a web of interconnecting filaments extending throughout most of the cytoplasmic space, when anaesthetic is present this web-like arrangement adopts a more strand-like appearance resulting in a more irregular distribution of these filaments throughout the cytoplasm (figure 5.10(b)). When control cells are heated at $43°C$ there is very little evidence of the intermediate filament network (figure 5.10(c)) suggesting that this cytoskeletal component is disrupted by the heating process. The small amount of IF that remains appears to be associated with the cell boundaries and nuclear region. Whilst there is no evidence of intermediate filament structure in the dibucaine and heat-treated cells (figure 5.10(d)) it is clear that the extraction processes have not worked very successfully in this instance. However, the fact that cytoplasmic material has collapsed onto the nuclear structures seems to suggest a total breakdown of the internal skeletal framework of which the intermediate filaments are an important part.

5.4 Discussion

From the results it would appear that the presence of tertiary amine local anaesthetics can influence cell survival at normal culture temperatures (figure 5.2), can potentiate hyperthermic cell death at $43°C$ (figures 5.3 to 5.5 and Table 5.1), can alter membrane 'fluidity' (figures 5.6 and 5.7), can affect surface morphology changes that take place at elevated temperatures (figure 5.8) and

may exert an effect on the intermediate filament network present in cells at normal culture temperatures (figure 5.10).

Before comparisons are drawn between the findings of these studies and those of other workers it is important to consider the idea of membrane 'fluidity'. As discussed in Chapter 4, membrane 'fluidity' is a rather poorly defined term, with different workers having their own interpretations. In the present study, following the suggestion of Stubbs and Smith (1984), membrane fluidity has been interpreted in terms of the physical state of the fatty acyl chains of the phospholipids comprising the membrane bilayer structure. This 'fluidity' has been measured through the insertion of the fluorescent polarisation probe, DPH, into membranes which is thought to measure the angular range of motion (degree of order) of the acyl chains (Lepock, 1982). However, as Yatvin et al. (1982) pointed out, since such probes only see the average properties of the whole system, uncertainties can arise with respect to the subcellular distributions of the probe, its lateral distribution between adjacent domains (e.g. gel and liquid crystalline phases) and its vertical distribution within the bilayer matrix. Work by Jain and Wu (1977) on the interaction of small molecules with artifical phospholipid vesicles has led Yatvin to dispute the fluidity measurements made by Lepock et al. (1981) which suggested a lack of correlation between hyperthermic cell killing and membrane lipid fluidity, since these workers assumed that the spin label 2,2-dimethyl-5-dodecyl-5-methyloxazolidine-N-oxide (2N14) exhibited isotropic behaviour throughout the thickness of the bilayer which Jain and Wu have shown is not possible.

The fact that 'fluidity' is characterised by a large number of parameters, which may be measured in many different ways, has undoubtedly led to some of the confusion that exists in the literature over the way it is affected by factors such as heat and anaesthetics. For example, when Yau (1979) working with 3T3 mouse fibroblast and murine L5178Y lymphoma cells measured fluidity in terms of fluorescence polarisation with perylene as the probe, he observed an increase in fluidity in the presence of procaine. However, when DPH was used as the probe a slight decrease in fluidity was observed. Yau interpreted the results as suggesting that the DPH and perylene probes embedded in entirely different membrane subregions with procaine significantly increasing the fluidity of the

membrane region where perylene equilibrated. However, it could be that the difference in results was due to fluorescence quenching, since direct quenching of DPH has been reported with procaine in model membranes (Surewicz and Leyko, 1982). Likewise when Constantinescu et al. (1986) were looking at the effects of procaine on membrane fluidity in erythrocytes, using the relative rate of haemolysis as a parameter of fluidity, they found that membrane fluidity decreased with increasing anaesthetic concentration. Whilst the results obtained with dibucaine and tetracaine in the present study support the commonly held view that membrane fluidity is increased by local anaesthetics, the results obtained with procaine do not. However, since Sweet and Schroeder (1986) using the same DPH probe reported a fluidising effect of procaine ($10mM$) with LM fibroblast plasma membranes, it could be that the single result obtained in this study has been influenced by fluorescence quenching effects.

Potentiation of hyperthermic cell killing by the presence of local anaesthetics as observed in the present study with certain concentrations of the anaesthetics dibucaine, tetracaine and procaine, has been reported by several workers. Yatvin (1977) working with the bacterium *E.coli*, showed that the cytotoxic effect of heating was enhanced when heating was conducted in the presence of procaine-HCl, whilst Yatvin et al. (1979) employing the local anaesthetic lidocaine reported that tumour bearing mice treated with heat and local lidocaine injection survived significantly longer than tumour bearing animals treated with heat or anaesthetic alone. Barker (1985) has confirmed Yatvin's findings in tumour bearing rats which were treated with heat and/or local injections of tetracaine. Yau (1979) working with a variety of cultured cells found that procaine potentiated hyperthermic killing at $43°C$ in a dose dependent manner. More recently Coss and Dewey (1988) have shown that in addition to sensitising asynchronous mammalian cells, procaine can also sensitise synchronous populations of CHO cells to hyperthermic cell death at $43°C$.

The correlations found in this study between the anaesthetic potency of dibucaine, tetracaine and procaine and their effects on cell survival at $43°C$, are in agreement with the findings of Yatvin et al. (1982) in his studies with *E.coli* and the local anaesthetics lidocaine, procaine and tetracaine.

The fact that all three local anaesthetics at relatively high concentrations produced a dramatic decline in cell survival at $37°C$ is thought to be due to the detergent like action of anaesthetics (Fernandez, 1980; Maher and Singer, 1984). Early studies by Fernandez (1980) with amphipaths such as chlorpromazine and tetracaine suggested that it was only at very high concentrations exceeding their critical micelle concentration (cmc), typically 60-70mM for tetracaine, that such amphipaths disrupted membranes and caused at least their partial dissolution through the formation of mixed micelles containing the amphipath along with the particular membrane component. However, Maher and Singer (1984) have shown that even at concentrations which are still relatively high but are below their cmc (typically 0.5mM for tetracaine) the effects of amphipaths on membranes may still reflect the detergent-like affinities for membrane components that the amphipaths exhibit in mixed micelle formation at very high concentrations.

The surface morphology changes that were observed with normal cells during hyperthermic treatment during this study have also been reported by a number of workers involved both with monolayer cultures (Lin et al., 1973; Bass et al., 1978; Schamhart et al., 1984) and suspension cultures (Kapiszewska and Hopwood, 1986).

Although the reduction in the number of microvilli and increased bleb formation reported by a number of workers (Bass et al., 1978; Kapiszewska and Hopwood, 1986) was not observed with control cells after 1 hour of heating at $43°C$, this probably reflects the length of time that the cells were exposed to the heat and the temperature. Bass et al. (1978) exposed CHO cells to $43°C$ for 3 hours whilst Kapiszewska and Hopwood (1986) exposed CHO cells to a range of temperatures $41.5°C$, $43.5°C$ and $45.5°C$ for various times and found that cells exposed to lower temperatures exhibited less blebbing, whilst in cells exposed to $45.5°C$ the percentage of cells with blebs following heating was dependent on the duration of heating with increases from 40% for 5 minutes to 90% for 30 minutes.

Whilst exposure of cells to dibucaine at $37°C$ caused a slight change in morphology, with cells tending to adopt a more rounded morphology, exposure of cells treated with dibucaine to heat at $43°C$ for 1 hour produced very different and marked morphological changes to the cell surface as compared to control cells

heated for the same period of time. There were no microvilli present and the cell surface was characterised by a large number of pits. Mulcahy et al. (1981) have reported a similar modification in the morphological response of P388 ascites tumour cells in the presence and absence of procaine at $37°C$ and $43°C$. Yau (1979) observed that in the presence of $30mM$ procaine 3T3 mouse fibroblasts rounded up within 10 to 15 minutes at $37°C$. He also observed the occurrence of smooth blebs similar to those seen on the plasma membrane of hyperthermia treated cells which suggested that heating or treatment of cells with local anaesthetic might exert a certain type of membrane derangement via a similar mechanism. Clearly if the acquisition of such similar membrane morphology generally precedes the inactivation of cells this could explain the potentiating effect of anaesthetics in the presence of heat.

The morphological findings of the current study together with the effects of the anaesthetic dibucaine on membrane order (figures 5.6 and 5.7) and on cell survival at $43°C$ (figure 5.3) clearly seem to suggest a couple of points. For example, since dibucaine has been shown both to fluidise plasma membranes relative to control membranes and to potentiate hyperthermic cell death it would seem that membrane 'fluidity' could well be a key factor determining the response of cells to heat. In addition, the marked morphological changes that were associated with this increase in cell death in the presence of dibucaine would seem to suggest that the mechanism of hyperthermic cell killing could well involve damage to the plasma membranes.

Many studies have been made on the intermediate filament networks present in cells such as hepatocytes (Franke et al., 1979; French et al., 1982; Ishii et al., 1985), macrophages (Phaire-Washington et al., 1987) and in cultured cells such as Madin Derby Canine Kidney (MDCK) cells (Fey et al., 1984), prostatic carcinoma DU 145 cells (Chakraborty and Von Stein, 1986), Baby Hamster Kidney (BHK) and 3T3 mouse fibroblast cells (Goldman et al., 1986). Such studies have revealed that intermediate filaments (IF) are typically $10nm$ in diameter and form a rich interconnecting network throughout the cytoplasm of cells extending from the nucleus to the cell surface. Goldman et al. (1986) have suggested that this IF system and the proteins that have been found to be associated with it, may well represent a chain of molecular connecting links between the nucleus

and cell surface. The interaction of IF with other structures in the cytoplasm, such as microtubules and centrioles but in particular with the plasma membrane has been observed in many cell types. For example, association between IF and plasma membranes in epithelial cells where keratin filaments have been observed to interact with desmosomal junctions is well documented (Schliwa, 1986). Similarly IF have been shown in close proximity in chicken embryo fibroblasts (CEF) to the microfilament bundles that accumulate subadjacent to the plasma membrane in specialised regions known as the fibronexus (Green and Goldman, 1986). Thus it seems reasonable to presume that agents such as tertiary amine local anaesthetics that act at a membrane level may well have a secondary effect on the IF network of cells.

Fey and Penman (1984) working with MDCK cells and a range of tumour promoters such as phorbol 12-tetradecanoate 13-acetate (TPA) and mezerein have shown that the significant change in cell surface morphology that results is reflected by a profound re-organisation in the NM-IF, suggesting that the information for architectural expression is mediated by the skeletal networks of each cell. The results obtained in the current study with dibucaine at $37°C$ would seem to support this idea since the more rounded morphology that was adopted by cells was accompanied by a more irregular distribution of the IF throughout the cytoplasm.

A number of workers have reported the collapse of IF on heating. Welch and Suhan (1985) working with rat fibroblasts showed that at $37°C$ the IF and microtubules showed a well spread and often coincident distribution. However, after exposure to $42°C$ for 3 hours, whilst no obvious change was observed in the distribution of microtubules, the IF collapsed and were found to aggregate around the nucleus. A similar finding was reported by Collier and Schlesinger (1986) who were working with chicken embryo fibroblasts. Whilst no gross morphological changes occurred in cytoplasmic microfilaments and microtubules exposed to $45°C$ for 3 hours, the IF network was found to be very sensitive, again collapsing around the nuclei of cells. Thus the findings of the current study are in keeping with other workers, but suggest that whilst dibucaine may have an important effect on the IF network at normal culture temperatures, at elevated temperatures

the breakdown of the IF network may simply be the result of cellular stress since this occurs in the presence or absence of anaesthetic.

In order to be able to interpret the effects of the 3 local anaesthetics used in this study it is important to have an understanding of their site(s) of action and their molecular mode of action. However, although it is widely accepted that local anaesthetics produce their effects by interaction with membranes (Seeman, 1972), not only is the molecular mechanism of anaesthetic action not fully understood but also the site of action is often disputed.

Various investigations have presented evidence favouring either protein or lipid as sites of action. The isolation and purification of a protein which specially binds tertiary amine local anaesthetics from mammalian axonal membranes strongly supports the hypothesis according to which a protein should be a local anaesthetic receptor (Greenberg and Tsong, 1984). Similarly Chan and Wang (1984) have provided evidence of an interaction between local anaesthetics and membrane proteins. The variability of the chemical structure of various local anaesthetics, on the other hand, has led some workers to propose the possibility of heterogeneous sites of anaesthetic action which could involve binding sites on the protein and/or in the lipid matrix (Kelusky et al., 1986).

However, at the current time many studies, including the fluidity measurements of the present study, would seem overwhelming to favour lipid as the site of anaesthetic action. Early studies by Seeman (1972) showed that drug-lipid interactions correlated with anaesthetic potency. Further support for lipids being the initial target for anaesthetic action comes from the studies of Yatvin (1977) and Yau (1979) in which anaesthetic agents enhanced thermosensitivity. Although the hydrophobic areas of the membrane bound protein could conceivably be a site of anaesthetic action, the thermosensitivity of solid tumours was not further influenced by lidocaine in host animals fed diets enriched in linoleic acid, a diet which markedly modifies fatty acid and phospholipid patterns and cholesterol concentration of cellular membranes (Yatvin et al., 1983). This lack of effect would therefore seem to rule out a meaningful lidocaine-protein interaction.

Although membrane lipid would appear to be the likely site of anaesthetic action, the precise molecular mechanism involved in this interaction is still un-

clear. Part of the problem, already mentioned, is that many local anaesthetics are tertiary amine compounds. These tertiary amine compounds can exist in two forms in the aqueous solution (cationic and neutral) with the distribution of the two forms in the solution depending on the pKa of the anaesthetic as well as on the pH of the bathing solution. For example, Boulanger et al. (1981) showed in a deuterium/P^{31} N.M.R. study that tetracaine exerted different effects on phospholipid dispersions depending on whether it was positively charged (at pH 5) or uncharged (at pH 9.5). Also since local anaesthetics are amphipathic molecules they demonstrate complicated adsorption properties.

In an attempt to elucidate the molecular mechanism of anaesthetic action many workers have employed model membranes in their studies. For example the location of the local anaesthetic tetracaine in model membranes of phospholipids and its effects on the order and dynamics of the lipids have been the subject of several studies. Such studies have revealed that tetracaine decreases the order of lipid acyl chains and interacts differently with different phospholipids such as phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine depending mostly on the charge and the shape of the lipid studied (Smith and Butler, 1985).

Work by Kuroda and Fujiwara (1987), using unilamellar phosphatidylcholine vesicles and the cationic forms of procaine, tetracaine and dibucaine, has suggested that the anaesthetic potency of these drugs, which was found to reflect their ability to potentiate hyperthermic cell death in the current study, might not be correlated with their ability to affect membrane fluidity, but rather with their ability to bind to lipids at the polar head group of the bilayer. Procaine was found to interact very weakly with lipids at the outer surface of the vesicles, tetracaine was found to bond to the lipids both at the outer and inner halves of the bilayer inserting its rod-like molecule in a sea of acyl chains of phosphatidylcholine, whilst dibucaine bound tightly to the polar head group of phosphatidylcholine which only resided at the outer half of the bilayer vesicles. These workers suggest that tetracaine might serve to perturb the organisation and mobility of the lipid bilayer more effectively than dibucaine due to its molecular shape (see figure 5.1). Since tetracaine is a more cylindrical molecule it is proposed to penetrate more deeply into the lipid bilayer than dibucaine, acting as

a wedge to separate the phosphatidylcholine molecules from each other. This in turn will weaken both the electrostatic binding at the polar head groups and the hydrophobic interaction at the acyl chains among phosphatidycholine molecules, thereby increasing their mobilities. Dibucaine on the other hand, being a more bulky molecule, is proposed to bind shallowly with the lipids at the outer surface of the bilayer developing a positive surface charge effectively stabilising the bilayer vesicles and acting as a clamp that grasps the phosphatidylcholine molecules thereby reducing their mobility. These workers also suggested that the fluidising effects of dibucaine on multilamellar vesicles of phosphatidylcholine reported by Papahadjopoulos et al. (1975) at pH 7.2-7.4 could be due to a deeper penetration of its neutral form.

Singer and Jain (1980) have investigated the interactions of dibucaine, procaine and tetracaine at various temperatures with multilamellar liposomes composed of saturated phosphatidylcholines and dicetyl phosphate, with respect to their influence on sodium-22 efflux. The liposomes were found to display a permeability maximum in the temperature region of their phase transitions which could be increased and caused to occur at a lower temperature in the presence of these anaesthetics. The order of potency of these anaesthetics in terms of their capacity to alter ^{22}Na permeability was found to be dibucaine > tetracaine > procaine, with procaine having no effect on the permeability properties. They also found a linear relationship between aqueous anaesthetic concentrations causing equal permeability effects and the corresponding concentration of the anaesthetics in the membrane. In addition, the permeability effects of the local anaesthetics were modulated by the length of the lipid fatty acyl chains and the anaesthetics were found to bind preferentially to the boundary regions between gel and liquid crystalline lipid found at the transition temperature.

Ohki (1984), in a study with the cationic forms of dibucaine, tetracaine and procaine and vesicles composed of phosphatidylcholine or phosphatidylserine demonstrated that the surface adsorption order was dibucaine > tetracaine > procaine in accordance with the reported anaesthetic potency, and that the amounts of local anaesthetic adsorbed on phosphatidylserine membranes was much greater than that of the phosphatidylcholine membrane. He also found that Ca^{2+} could affect the adsorption of the charged form of local anaesthetics

onto the membrane. The adsorption of anaesthetic molecules onto the membrane surface was suppressed in the presence of Ca^{2+} in the solution in the region of low concentrations of local anaesthetics. However this inhibitory effect was removed at higher anaesthetic concentrations. This finding was of interest since Ca^{2+} binding to membranes has been observed to make the membrane more rigid (Gordon et al., 1983). Hence the fact that local anaesthetics can compete with Ca^{2+} for binding sites at higher concentrations offers one explanation for the way in which these agents bring about their disordering effect on membranes.

Although studies with model membranes have revealed some of the mechanisms by which anaesthetics may exert their actions, when natural membranes are considered the picture becomes more complex. For example, in addition to phospholipids, membranes also contain a relatively large amount of cholesterol. Studies on the interactions of the local anaesthetic tetracaine with phosphatidylcholine membranes containing a physiological concentration of cholesterol (Auger et al., 1988) have suggested a different location of the anaesthetic in cholesterol containing systems compared to that of pure phosphatidylcholine bilayers. The tetracaine molecule has been shown to sit higher in the membrane, closer to the aqueous interface of the lipid bilayer than is suggested by the studies of Kuroda and Fujiwara (1987).

In addition it is known that the lipids of the cell surface membrane are not symmetrically distributed across the membrane bilayer (Op den Kamp, 1979) which means that charged amphipaths such as anaesthetics may also preferentially insert into one leaflet of the bilayer thereby selectively fluidising that leaflet (Sheetz and Singer, 1974). Sweet and Schroeder (1986) have demonstrated selective fluidisation by local anaesthetics of individual leaflets in isolated LM-fibroblast plasma membranes. Thus the functional consequences of alterations in membrane fluidity that are brought about by local anaesthetics will depend in part on which leaflet or leaflets they fluidise. Whilst Sweet and Schroeder (1986) propose that procaine fluidises both leaflets, at least in LM-fibroblast plasma membranes, other workers (Dipple et al., 1982) have suggested that most cationic local anaesthetics such as tetracaine, bind preferentially to the inner leaflet.

Clearly the molecular mechanism of anaesthetic interaction with membranes is highly complex and dependent on many factors. Although the evidence cited above would seem to suggest an anaesthetic-lipid interaction causing an increase in membrane 'fluidity', the effect of any local anaesthetic will depend not only on its molecular shape, size and adsorption properties but also on the distribution of lipids in the cell surface, the possible hydrophobic and electrostatic interactions, its concentration and whether it is present in the cationic or neutral form. Such factors will in turn influence the extent to which anaesthetic molecules can alter the structure of membranes and in particular the membrane 'fluidity' which would appear to be one of the most important influences when considering the factors that affect the hyperthermic cell death of HTC cells.

General Discussion

The aim of this thesis has been to explore the hypothesis that the damaging effects of hyperthermia on cells acts at the plasma membrane, and that bilayer lipid composition and physical state are critical features in the expression of heat damage.

In any study that monitors the response of cells to elevated temperatures there is the problem of artefact responses that arise simply as a consequence of changing environmental and assay conditions. Consequently, much of the work presented in Chapters 2 and 3 was concerned with standardising cell culture conditions and techniques for the chosen Hepatoma Tissue Culture (HTC) cell line.

One of the major advantages of the cell culture system is that it offers the opportunity to control physicochemical and physiological conditions very easily. Initial studies revealed that pH variations occurred at elevated temperatures, particularly in 24-well plates. In addition, fungizone and reduced levels of serum were found to increase the sensitivity of HTC cells exposed to elevated temperatures. Consequently, Hepes buffer ($10mM$) was incorporated into heating medium to minimise pH fluctuations and fungizone was omitted from heating medium. A consistent 10% (v/v) level of serum was maintained in heating medium. Adoption of such measures ensured the feasibility of comparative studies, and also ensured that the effects observed in subsequent experiments with cells treated with fatty acids or local anaesthetics were due to heat and were not artefactual.

The work reported in Chapters 2 and 3 also assessed two alternative methods for assaying cell survival following heat treatment. Since one of the key features of a cancer cell is its unlimited capacity for proliferation, a relevant endpoint

to consider in terms of the effectiveness of any treatment is loss of reproductive ability. The traditional clonogenic assay, which relies on the ability of a surviving cell to give rise to a colony, provided a fairly unambiguous definition of survival. The major drawback of this technique was the time involved with HTC cells, for they typically required a 9 day post-heating incubation period, and several media changes, for reasonable colony formation. However, the alternative colorimetric assay that was developed in the current study, whilst providing a much quicker estimate of cell survival, has produced survival curves that differ markedly from those obtained with the clonogenic assay following heat treatment.

The clonogenic assay survival curves generated for HTC cells over the hyperthermic temperatures $43°C$ to $45°C$ were characterised by an initial shoulder region followed by a phase of logarithmic cell killing. The fact that the shoulder region of these curves decreased as the temperature was increased supports the concept that the shoulder region of the survival curve reflects the cells' ability to sustain sublethal damage (Hahn, 1982). However, the survival curves produced by the colorimetric assay at elevated temperatures were very different, often lacking the initial shoulder. In addition, at temperatures of $42.5°C$, $43.5°C$ and $44°C$ the colorimetric assay survival curves were biphasic in nature and suggested an enhanced cell survival relative to the clonogenic assay at longer exposure times.

A consideration of the literature suggests that the differences observed between the clonogenic assay and colorimetric assay could reflect the different parameters used to assess cell survival (reproductive activity versus metabolic activity) and also differences in the time after heating at which the assays were performed (10 days versus 3 days), since a cell may lose its reproductive ability but still maintain some metabolic activity. Also, Vidair and Dewey (1988) working with CHO cells heated in the plateau phase of growth have reported a 'rapid' mode of death which was evident during the first few days post heating and a 'slow' mode of death that occurred typically 6 or 7 days post heating. In the current study it is proposed that since the colorimetric assay was conducted at 3 days post heating, rather than 6 or 7, it failed to account for cells dying by the slow mode of death. Assuming that the proportion of cells affected by the slow mode of death is fairly small, this would explain why such cells have a fairly insignificant effect at shorter time points. However, at longer time points,

where fewer cells survive, the contribution from these cells will become significant thus accounting for the biphasic survival curves observed at temperatures between $42.5°C$ and $44°C$. The linearity of the $45°C$ colorimetric survival curve is thought to suggest a decline in cell death by the slow mode of death as has been suggested to occur by Vidair and Dewey (1988) as the temperature is increased.

The development of a suitable cryopreservation procedure for these HTC cells provided a safeguard against loss of cells by contamination and also meant that any changes in cell characteristics that can occur when cells are continuously grown in an *in vitro* environment (Coriell, 1976) could be minimised by re-initiation of the cell line from a frozen cell suspension at intervals during the course of the study.

The morphological studies that are presented in Chapter 3 support the hypothesis that the plasma membrane is involved in the hyperthermic death of HTC cells. The gradual loss of microvilli during heating and the formation of 'blebs' on the cell surface following 80 minutes of heating at $43.5°C$ clearly indicates that membrane morphology is undergoing a change as a result of exposure to heat. The fact that the appearance of blebs on the cell surface coincides with the time at which a marked decrease in cell survival begins to be observed for these cells suggests the importance of this type of damage in hyperthermic cell death. Bass et al. (1982) and Borrelli et al. (1986) both working with CHO cells have also provided evidence for a correlation between membrane blebbing and cell death at hyperthermic temperatures ranging from $42°C$ to $45.5°C$.

Also in Chapter 3, the attempt to develop a suitable supplementation method for HTC cells has revealed that unlike LM cells that can be grown in serum-free medium (Konings, 1985) or 3T3 cells that can be grown in lipid-depleted serum (Horwitz et al., 1974), the HTC cells grown in this laboratory require the presence of unprocessed serum. In addition, whilst supplementation of HTC cells with the unsaturated fatty acid, linoleic acid (18 : 2) in medium containing 10% (v/v) serum levels does not substantially alter the growth of cells at concentrations up to $80\mu M$ fatty acid, cell growth is markedly inhibited in the presence of $20\mu M$ stearic acid (18 : 0), a saturated fatty acid. Similar effects with various saturated and unsaturated fatty acids have been reported by a number of

investigations utilising different cell lines (e.g. Doi et al., 1978; Spector et al., 1979). The accumulation of lipid droplets in the cytoplasm of HTC cells in the presence of linoleic acid supplement and their subsequent disappearance when cells were returned to normal culture medium has been reported by a number of workers (Geyer, 1967; Schneeberger, 1971). The presence of lipid droplets in the cytoplasm of HTC cells following supplementation highlighted the importance of producing purified plasma membrane fractions for lipid fluidity measurements. A number of workers (e.g. Collard and De Wildt, 1978; Stubbs et al., 1980) have shown that fluidity measurements conducted on whole cells that contain lipid droplets can yield misleading results due to the incorporation of the probe within the cytoplasmic droplets.

HTC cells supplemented with $60\mu M$ linoleic acid (18 : 2) showed enhanced thermal sensitivity at $43°C$ following a 36 hour period of supplementation. The period of supplementation appeared to be important since this increase in thermal sensitivity was not apparent after either a 24 or a 48 hour period of supplementation. It seems likely that part of the reason for this is that the extent of fatty acyl chain modification of membrane lipids depends on the time of exposure to the supplemented fatty acid. Spector and Yorek (1985), for example, have shown that when Y79 retinoblastoma cells are exposed to $30\mu M$ docosahexenoic acid (22 : 6) for up to 72 hours, the greatest change in phospholipid fatty acyl composition occurs during the first 48 hours.

Ladha et al. (1989), working in this laboratory, have recorded a much greater increase in thermosensitivity of these HTC cells following a 36 hour period of supplementation with $60\mu M$ arachidonic acid as opposed to $60\mu M$ linoleic acid. Arachidonic acid reduced the LD_{90} value recorded at $43°C$ for supplemented cells by 30 minutes relative to control cells as opposed to the 10 minute reduction obtained with $60\mu M$ linoleic acid under identical experimental conditions. Part of the reason for this increase in thermal sensitivity following exposure of HTC cells to arachidonic acid may well be due to the higher level of unsaturation of this fatty acid as compared to linoleic acid since arachidonic acid is characterised by 4 unsaturated bonds as opposed to the two found in linoleic acid. In addition, it could also result from the higher degree of incorporation of this fatty acid into plasma membrane phospholipids. Fatty acid analysis of plasma membranes

derived from HTC cells that had been exposed to arachidonic acid or linoleic acid for a 36 hour period revealed that archidonic acid rose from a barely detectable level in control cell plasma membrane phospholipids to approximately 20% of the total fatty acid content (Ladha et al., 1989), whilst linoleic acid produced a 14% increase from 2% of the total fatty acid content to 16%. The results of Ladha et al. (1989) are comparable to the studies of Konings (1985) who demonstrated a marked increase in the thermal sensitivity of mouse fibroblast LM cells exposed to $44°C$ following supplementation with $100\mu M$ arachidonic acid (20 : 4) for a 24 hour period.

In Chapter 4, plasma membrane-enriched fractions were obtained from control cells and linoleic acid supplemented cells that had been grown by microcarrier culture and exposed to $60\mu M$ linoleic acid for a 36 hour period. The adoption of microcarrier technology, as described in Chapter 2, where HTC cells were grown as monolayers on small spheres kept in suspension by stirring, enabled HTC cell production to be scaled up in order to generate sufficient cells for purification of plasma membranes. However, the lower than expected yields of HTC cells produced from microcarrier culture under the present conditions suggest that future studies should attempt to optimise microcarrier culture conditions. The isolation procedure that has been developed involving self-forming Percoll gradients as compared to the more conventional sucrose density gradients (Lopez-Saura et al., 1978) produced plasma membranes that were purified approximately 15-fold with a recovery of 29% based on the assay of the plasma membrane enzyme Na^+/K^+ATPase. These results compare favourably with figures quoted by other workers. For example, Tweto et al. (1976) using a sucrose gradient separation reported a 12-fold purification of HTC plasma membranes and a much lower yield of 1.2% based on the assay of 5′nucleotidase as the plasma membrane marker. Amende and Donlon (1985), using a single Percoll gradient centrifugation step, reported a 4.5-fold purification of rat liver plasma membranes based on the assay of 5′nucleotidase. Sauvage et al. (1981) working with HTC cells and utilising sucrose density gradients and digitonin reported a 21-fold purification of plasma membranes, which was higher than the current study, but a similar yield (22.8%).

The degree of purity and yield of plasma membrane for any given cell type would seem to depend not only on the procedure used to isolate the plasma mem-

branes but also on the component used as the plasma membrane marker. The importance of selecting a marker that is specific for the plasma membrane has been highlighted by results of the present study. Whilst Na^+/K^+ATPase and adenylate cyclase assays both suggested a 20-30% yield of plasma membranes that were purified approximately 12 to 16-fold, data for alkaline phosphodiesterase I and cholesterol suggested a much lower yield at 10-13% with a 5 to 6-fold purification. Consideration of the literature suggests that alkaline phosphodiesterase I and cholesterol may not be ideal plasma membrane markers (see Draye et al., 1987; Amar-Costesec et al., 1974; Henning and Heidrich, 1974). Similarly, whilst Tweto et al. (1976) utilised 5′nucleotidase as a plasma membrane marker in HTC cells, the presence of this enzyme was not detected in the present study nor has it been detected by Lopez-Saura et al. (1978) which raises doubt as to its reliability as a plasma membrane marker for HTC cells.

Contamination of the final plasma membrane preparation produced in this study by lysosomes, mitochondria and endoplasmic reticulum appeared to be very low though further studies would be required to confirm this result since only two assays of each contaminant were possible in the time-scale available.

Isolation of plasma membranes from cell suspensions of both normal and linoleic acid supplemented HTC cells with similar levels of purity enabled comparative studies of membrane structure and function to be carried out. These studies in which the fatty acyl composition of phospholipids and physical state of plasma membranes were examined, strongly suggested that the plasma membrane and in particular its lipid composition and level of 'fluidity' could be an important factor in the hyperthermic death of HTC cells.

Supplementation of HTC cells with $60\mu M$ linoleic acid for 36 hours produced a significant decrease in the level of oleic acid (18 : 1) in plasma membrane phospholipids from 40% to 25% and a significant increase in the level of linoleic acid from 2% to 16%. Similar data has been obtained by King and Spector (1978) working with Ehrlich ascites cells. The proportion of saturated fatty acids in plasma membrane phospholipids, however, remained fairly constant at approximately 50% of total fatty acyl groups, suggesting that the biological mechanisms that detect changes in the fatty acid content of membrane phospholipids are

particularly sensitive to the ratio of saturated:unsaturated fatty acid. This constancy in saturated fatty acid levels has been observed by Neudoerffer and Lea (1967) working with sarcoplasmic reticulum from turkey breast. These workers attempted to modify the saturated:unsaturated fatty acid ratio in phospholipids from turkey beast sarcoplasmic reticulum by feeding Broad Breasted White turkeys for 10 weeks on either a beef or fish diet. The beef diet was rich in saturated fatty acids such as palmitic (16 : 0) and stearic acid (18 : 0) whilst the fish diet was rich in polyunsaturated fatty acids such as arachidonic acid (20 : 4) and docosapentaenoic acid (22 : 5). It was found that enriching the diet in saturated fatty acids had no significant effect on the fatty acid composition of the membrane phospholipids and that the total content of saturated fatty acid remained constant at slightly less than 50%. In addition, whilst enriching the diet in polyunsaturated fatty acids did produce a marked change in the unsaturated fatty acid content of the membrane phospholipids with 20 : 5 and 22 : 6 levels being increased largely at the expense of 18 : 1 and 18 : 2, there was little change in the saturated fatty acids which again were found to remain fairly constant at approximately 50% of the total phospholipid fatty acyl groups.

Two further interesting observations in the current study were that there was no change in the chain length distribution of the plasma membrane phospholipid fatty acids and also there was no significant change in the membrane cholesterol to phospholipid ratio after supplementation of HTC cells with linoleic acid, factors which have been shown to be capable of altering fluidity (see Stubbs and Smith, 1984). Whilst Sweet and Schroeder (1988) working with LM fibroblasts, and King and Spector (1978) working with Ehrlich ascites cells also found no appreciable change in the cholesterol:phospholipid molar ratio of fatty acid of supplemented cells, Edwards-Webb and Gurr (1988) have reported higher cholesterol:phospholipid ratios (relative to control membranes) in erythrocyte plasma membranes derived from guinea pigs fed diets rich in linoleic acid. The precise reason(s) for these discrepancies in cholesterol:phospholipid ratios following supplementation either *in vivo* or *in vitro* are not certain. It could be that the discrepancies reflect differences in cells' abilities to make compensatory changes in their plasma membrane composition that enable them to maintain the constant plasma membrane physical state thought necessary for normal cell functioning. For example, in the present study where there was an increase in fatty acyl

unsaturation of plasma membrane phospholipids following supplementation of HTC cells with linoleic acid but no change in the cholesterol:phospholipid ratio, there was also a change in plasma membrane physical state as measured by DPH fluorescence polarisation. However, in the studies conducted by Edwards-Webb and Gurr (1988) where guinea pigs were fed linoleic acid enriched diets for a 3-5 month period, the diet induced increases in the acyl chain unsaturation which occurred in erythrocyte membranes did not produce any increase in fluidity which would seem to be due to the concomitant increase in the ratio of cholesterol to phospholipid, since cholesterol has been shown to decrease membrane fluidity via its interaction with membrane phospholipids (Poznansky et al., 1973).

In Chapter 4 the lower steady state fluorescence polarisation values obtained with plasma membranes derived from HTC cells that had been exposed to $60\mu M$ linoleic acid for 36 hours suggest that supplementation with this unsaturated fatty acid produces membranes that are less ordered (more 'fluid') than control membranes. It seems logical to predict that the lower order of the plasma membranes derived from supplemented cells relates to the differences observed in the plasma membrane lipid composition. Thus it would seem that, in the case of HTC cells, the substitution of 18 : 1 by 18 : 2 in the plasma membrane phospholipids of supplemented cells is responsible for the decreased order observed.

However, a consideration of the literature reveals that unsaturation and membrane 'fluidity' are unlikely to be related in the simple and direct manner suggested by this study. Whilst a number of other workers have reported that supplementation with unsaturated fatty acids produces cells with more fluid membranes (e.g. Wolters and Konings, 1984; George et al., 1983), other workers do not report any alterations in 'fluidity' (e.g. Poon et al., 1981; Edwards-Webb and Gurr, 1988). As mentioned above, it could be that part of the reason for discrepancies observed in 'fluidity' following supplementation relate to the cells' ability to show adaptive responses to dietary manipulations. Other possible reasons for discrepancies could relate to the level at which studies have been conducted and the techniques that have been used to measure 'fluidity'. One of the limitations of this study is that time did not permit a consideration of all the possible perturbations that could have been produced in the plasma membranes as a result of supplementation. Changes in phospholipid headgroup composition can have

an important consequence on 'fluidity'. For example, an increase in the ratio of phosphatidylethanolamine to phosphatidylcholine causes an increase in the steady state polarisation of DPH (Gilmore, 1979). Similarly, positioning of the unsaturated fatty acid on the glycerol backbone ($sn-1$ or $sn-2$) of phospholipids and the position of the double bond(s) along the fatty acyl chain can also have a considerable effect on the physical properties of the membrane, apart from the effect of a change in the level of unsaturation alone (see Stubbs and Smith, 1984). Ideally, any future studies should aim to conduct more detailed investigations by, for example, looking for changes in the phospholipid head group composition and by examining fatty acyl composition of individual phospholipid classes to obtain a more accurate picture of the precise effects of supplementation on the composition, structure and physical properties of the plasma membrane. In addition, studies employing a greater variety of polyunsaturated fatty acids would be useful. Furthermore, because of the complex nature of membrane 'fluidity' and the diversity of biophysical techniques that have been developed which utilise a variety of 'probes' that are intercalated into the bilayer to measure particular aspects of the fluid condition, future studies could be improved by the utilisation of a wider variety of the techniques available. For example, whilst steady state fluorescence polarisation spectroscopy indicates the extent of 'wobbling' of a probe over a nanosecond time-scale and hence provides information on the range of motion (degree of order) of phospholipid fatty acyl chains, ^2H-NMR spectroscopy provides specific information on the dynamics of hydrocarbon flexing. The probe motions measured by different techniques, therefore, are likely to occur over different time-scales and changes in one may not necessarily correlate with changes in another. Whilst Lepock et al. (1983) obtained similar results for 'fluidity' by measuring either Electron Spin Resonance of the probe 2,2-dimethyl-5-dodecyl-5-methyloxazolidine-N-oxide or fluorescence polarisation of the probe 1,6-diphenyl-1, 3,5-hexatriene in Chinese hamster lung V79 cell plasma membranes, this does not necessarily mean that these two different techniques will give the same results in different cell types. Cells from different origins are usually characterised by distinct plasma membrane compositions (Lee, 1985) which may influence the way in which probes are intercalated within the lipid bilayer. The use of different techniques to measure membrane 'fluidity' might explain

why Storch and Schachter (1984) employing steady state fluorescence polarisation reported an increase in fluidity in rat hepatocyte plasma membranes that had been shown to contain increased levels of monoenoic and polyenoic acyl chains relative to control plasma membranes following exposure of rats to fat free diets, whilst Poon et al. (1981) using electron spin resonance reported no significant increase in fluidity following a 20% increase in the level of 18 : 1 in the plasma membrane phospholipids of murine T lymphocyte EL4 tumour cells. In addition, many probe techniques including DPH fluorescence polarisation simply provide average information on 'fluidity' depending on the distribution of probe positions within the membrane even though the plasma membrane is now thought to be made up of a number of distinct microdomains each with different physical properties (Schroeder, 1983). As pointed out by Cossins and Raynard (1987), this means that probe techniques that give an overall average value for 'fluidity' will not reflect the state of any given region in the membrane so that the precise reasons for changes in membrane fluidity can not be determined. For example, a change in membrane average 'fluidity' may result from changes in the properties of probes in some microdomains but not others or it may result from a change in the entire population of probe molecules. Thus ideally, a range of probes sensitive to different regions of the bilayer should be employed to gain a better understanding of the importance of 'fluidity' in determining the response of cells to elevated temperatures.

It would also be of interest in future studies to investigate the composition and physical state of HTC cell plasma membranes at a wider range of time points following supplementation in an attempt to explain the change in thermal sensitivity of these HTC cells that was observed, for example, after a 48 hour period of supplementation.

However, the fact that changes in lipid composition were found to take place in membranes of supplemented HTC cells which were associated with an increase in 'fluidity', as measured by steady state fluorescence polarisation, and the fact that these changes were associated with an increase in thermal sensitivity of the cells, strongly supports the proposition that the lipid composition and level of fluidity of the plasma membrane play an important role in the response of cells to hyperthermia.

A further extension of this study could be to grow HTC cells at different temperatures, say $32°C$ and $39°C$ as opposed to the usual $37°C$ and then to monitor the response of these cells to hyperthermic temperatures and relate this to plasma membrane composition and physical state, since a number of workers have observed changes in plasma membrane composition of mammalian cells as a result of growth at elevated or lowered temperatures (e.g. Culver and Gerner, 1982). This would provide additional information on the way in which the modulation of membrane lipid composition and 'fluidity' are related to cellular thermal sensitivity. Though some work has been carried out in this area (Anderson et al., 1981; Culver and Gerner, 1982; Bates et al., 1985) the membrane fraction utilised has often been poorly defined and characterised which leads to problems in interpretation of results. Having developed a rapid and reliable method for isolating HTC cell plasma membranes in the current study, such studies with HTC cells could prove productive.

Since specific membrane functions are mediated by proteins and only indirectly by the properties of the lipid matrix of the membrane (Leyko and Bartosz, 1986), a number of workers have suggested that membrane proteins rather than membrane lipids are the main site of action. Lepock et al. (1981) found that butylated hydroxytoluene (BHT) fluidised membrane lipids of V79 Chinese hamster lung fibroblast cells, as measured by the lipophilic spin label 2,2-dimethyl-5-dodecyl-5-methyloxazolidine-N-oxide (2N14), but did not affect the inactivation rate of cells at $42.6°C$ and actually decreased the rate at $43.6°C$. However, Fukuzawa et al. (1981) reported that BHT rigidified rather than fluidised phosphatidylcholine liposomes as judged by DPH fluorescence polarisation. Analysis of data on the sensitisation to heat of V79 cells by alcohols has suggested that alcohols affect membrane proteins rather than lipids (Massicotte-Nolan et al., 1981). In the presence of low concentrations of alcohols the degree of heat sensitisation increased in the following order: methanol > ethanol > propanol > butanol. Whilst this order of sensitisation potency did not correlate with their fluidising abilities as measured by the electron spin resonance probe 2N14, it did correlate well with their effects on the temperature of thermal denaturation of a number of non-membraneous proteins. Similarly, ethylene glycol which did not affect the hyperthermic cell killing of V79 cells did not exert any clear cut influence on thermal denaturation of proteins, whilst glycerol, which protected the

cells from hyperthermia, elevated the thermal inactivation temperature of the proteins (Massicotte-Nolan et al., 1981). Recent studies by Lepock and collaborators (Lepock et al., 1988, 1989) have also stressed the importance of proteins in the hyperthermic killing process. Studies by Stevenson et al. (1981) with Chinese hamster ovary cells have also shown that heat induced alterations in the binding of hormone or antibody receptors correlate with heat sensitivity measured in terms of cell survival.

However, it is perhaps more likely that both lipids and proteins of the plasma membrane are involved in the hyperthermic response. It is known that exposure of cells to elevated temperatures serves to increase the molecular motion of membrane lipids and may well cause a change in the phase of lipids from a more ordered gel phase to a less ordered liquid crystalline phase (Lee and Chapman, 1987). Whilst the presence of proteins leads to non-uniformity in the viscosity of lipids it seems likely that proteins are themselves influenced by the dynamic state of the membrane lipids. It is thought that, on account of the anisotropic and hydrophobic nature of the lipid bilayer, enzymes probably need to possess relatively loose tertiary structures to enable the molecular flexibility that is vital for catalysis. This could also make such enzymes particularly sensitive to heat for such structures could be susceptible to perturbation. Hyperthermic temperatures that serve to increase the molecular motion of membrane lipids (decrease lipid order) would provide a less hindered environment for proteins. This would in turn permit a greater range of conformational movement on the part of the proteins and could result in proteins adopting configurations that are inactivating.

The studies of membrane associated enzyme activity (Chapter 4), which provides a natural 'probe' of the adjacent lipid region surrounding membrane proteins, suggested that alkaline phosphodiesterase I in HTC cell plasma membranes from linoleic acid-supplemented cells could be more thermolabile than the same enzyme from control membranes, although further studies would be required to confirm the significance of this data. These results support the idea that perturbation of the plasma membrane by heat has a destabilising effect on proteins present in the bilayer. In addition, the fact that the thermosensitivity of alkaline phosphodiesterase I can be potentiated by supplementation of HTC cells with

linoleic acid, which has been shown to cause an increase in plasma membrane fluidity relative to control membranes, suggests the importance of plasma membrane fluidity in determining the response of membrane proteins to heat. Barker (1985) working with rat liver and with MC7 sarcoma and D23 hepatoma, two transplantable rat tumour lines, has similarly reported greater thermosensitivity of the plasma membrane Mg^{2+}ATPase of tumour cells which correlated with a lower lipid order (increased plasma membrane fluidity). A correlation between plasma membrane cholesterol levels and the thermosensitivity of Ca^{2+}ATPase in reconstituted lipid vesicles has also been reported (Cheng et al., 1987), again suggesting the importance of lipid fluidity in the effects of heat.

The physical state of plasma membrane lipids can also be modulated by a range of 'membrane active' agents which include alcohols and local anaesthetics. Whilst it is generally accepted that local anaesthetics produce their effects by interaction with membranes (Seeman, 1972) which causes an increase in membrane 'fluidity' (Butler et al., 1973) their site of action and molecular mechanism of action is not well understood. Current evidence favours lipids rather than proteins as the site of anaesthetic interaction (see Chan and Wang, 1984), whilst other studies into the molecular mechanism involved in their fluidising effect have revealed the complexity of their action. The effect of local anaesthetics on plasma membrane lipids have been shown to depend on a wide range of factors including the molecular shape of the anaesthetic molecule (Kuroda and Fuijawara, 1987), its adsorption properties (Ohki, 1984) and whether it is in a cationic or neutral form (Boulanger et al., 1981).

The work presented in Chapter 5, utilising local anaesthetics in their cationic form, has shown that when HTC cells are heated in the presence of dibucaine, tetracaine or procaine there is a potentiation of hyperthermic cell killing, with the potentiation increasing with increasing concentration of the anaesthetic in the heating medium. Potentiation of heat death in the presence of anaesthetics has been reported by a number of workers (e.g. Yatvin, 1977; Yau, 1979; Bowler et al., 1987; Dewey, 1988). In the current study steady state fluorescence polarisation measurements, utilising DPH as the probe, also indicated that in the presence of increasing concentrations of dibucaine and tetracaine there was a

corresponding decrease in polarisation values measured in purified plasma membranes, implying a decrease in membrane order, i.e. increase in 'fluidity'. These results therefore provide evidence for the importance of increased membrane fluidity in the hyperthermic death of HTC cells.

Another interesting point that emerges from the anaesthetic work that was conducted in this study is that the increase in 'fluidity' of plasma membranes that was produced by adding dibucaine to HTC cells at $37°C$ is equal to the increase in fluidity of plasma membranes produced by exposing cells to $43°C$ (consult figure 5.6). However, only one condition (exposure of cells to $43°C$) resulted in cell death. This suggests that fluidity changes alone are not the direct cause of cell death. Rather, it would appear that lipid fluidisation serves to influence some important membrane component, in all probability membrane proteins, in a critical way, that leads to cell death.

However, the results obtained with procaine are difficult to explain. Whilst $5mM$ procaine produced a highly significant decrease in cell survival at $43°C$ relative to control cells, the same concentration of anaesthetic had no effect on the steady state DPH fluorescence polarisation values obtained. It could be that procaine increases hyperthermic cell death by a different mechanism to dibucaine and tetracaine, however, the fact that Sweet and Schroeder (1986) also using DPH as a fluorescence probe reported a fluidising effect of $10mM$ procaine with LM fibroblast plasma membranes, suggest that the procaine results of the current study could be questionable. The studies of Yau (1979) are of interest since he also reported a potentiation of hyperthermic killing in murine 3T3 and CHO cells following procaine treatment and very little effect on plasma membrane fluidity as measured by DPH fluorescence polarisation. However, when Yau used perylene as the fluorescent probe the fluidity of cell membranes of procaine treated cells was found to increase significantly. These observations taken together with the fact that the presence of procaine, at least in model membranes, can result in fluorescence quenching (Surewicz and Leyko, 1982) suggest that alternative measurements of membrane fluidity should be conducted on purified HTC plasma membranes in the future in the presence of procaine, before it is assumed that this anaesthetic does not alter membrane fluidity.

The morphological changes observed on heating cells in the presence of the anaesthetic dibucaine provide further evidence for the important role of membranes and membrane fluidity in hyperthermic cell death. HTC cells heated in the presence of dibucaine, which produced an increase in membrane fluidity, were characterised by more extreme surface morphological changes than control cells heated in the absence of anaesthetic. Microvilli were lost from the membrane surface, which in turn was characterised by a high degree of deformation in the form of pits in the cell surface. Similar findings have been reported by Mulcahy et al. (1981) working with P388 ascites tumour cells in the presence and absence of procaine at $37°C$ and $43°C$ and would seem to suggest that a cell's response to hyperthermia may well be related to its membrane fluidity at the time of treatment.

Studies on the Intermediate Filament (IF) network of HTC cells (Chapter 5) indicated that under normal culture conditions cells are characterised by a web of interconnecting filaments extending throughout the cytoplasm. Exposure of cells to dibucaine, however, appeared to produce a more irregular distribution of IF within the cytoplasm, which could account for the rounded morphology of HTC cells observed in the presence of dibucaine at $37°C$. These results suggest that local anaesthetics such as dibucaine that are known to act at the plasma membrane level may well produce a secondary effect on the IF network of cells at $37°C$. However, since heating both control and anaesthetic treated cells produced similar disruption of the IF network in HTC cells, it seems unlikely that IF damage is a primary factor determining the differential response of cells to elevated temperature in the presence or absence of anaesthetics.

In conclusion, the work presented in this thesis supports the concept that the plasma membrane is an important site of hyperthermic damage and that its lipid composition and physical state play a major role in determining the response of cells to hyperthermic insult, possibly by modulating membrane protein thermosensitivity.

It is proposed that exposure of cells to elevated temperatures results in a decrease in plasma membrane order (increase in fluidity) which in turn leads to changes in protein-lipid interactions and the adoption of configurations by

membrane proteins that are inactivating. Such heat damage to the plasma membrane is thought to lead to changes in membrane permeability and to a loss of membrane receptor and membrane enzymatic activity. These alterations would inevitably lead to a loss of cellular homeostasis and ultimately to cell death.

References

ALAM, S. Q. & ALAM, B. S. (1986) Effect of essential fatty acid deficiency on acyl group composition, membrane fluidity and adenylate cyclase in plasma membranes of rat submandibular salivary glands, *J. Nutr.* **116** 1620–1630.

ALANIZ, M. J. T. de., PONZ, G. & BRENNER, R. R. (1975) Biosynthesis of unsaturated fatty acids in cultured minimal deviation hepatoma 7288C cells, *Acta Physiol. Latinoam.* **25** 1–11.

ALANIZ, M. J. T. de., GOMEZ DUMM, I. N. T. de. & BRENNER, R. R. (1976) The action of insulin and dibutyryl cyclic AMP on the biosynthesis of polyunsaturated acids of α-linoleic acid family in HTC cells, *Mol. Cell. Biochem.* **12** 3–8.

ALANIZ, M. J. T. de., GOMEZ DUMM, I. N. T. de. & BRENNER, R. R. (1982) Effect of different carbon sources on $\Delta 5$ desaturation activity of HTC cells, *Mol. Cell. Biochem.* **48** 15–18.

ALANIZ, M. J. T. de., GOMEZ DUMM, I. N. T. de. & BRENNER, R. R. (1984) Effect of fatty acids of $w6$ series on the biosynthesis of arachidonic acid in HTC cells, *Mol. Cell. Biochem.* **64** 31–37.

ALBERTS, B., BRAY, D., et al. (1983) *Molecular Biology of the Cell*, Garland publishing, New York.

ALLEY, M. C., SCUDIERO, D. A., MONKS, A., CZERWINSKI, M.J., SHOEMAKER, R. H. & BOYD, M. R. (1986) Validation of an automated microculture tetrazolium assay (MTA) to asses growth and drug sensitivity of human tumour cell lines, *Proc. Am. Assoc. Cancer Res* **27** 389.

ALLEY, M. C., SCUDIERO, D. A., MONKS, A., HURSEY, M. L., CZERWINSKI, M. J., FINE, D. L., ABBOTT, B., MAYO, J. G., SHOEMAKER, R. H. & BOYD, M. R. (1988) Feasibility of drug screening with panels of human tumour cell lines using a microculture tetrazolium assay, *Cancer Res.* **48** 586–604.

ALLISON, A. C. & PATON, G. R. (1965) Chromosome damage in human diploid cells following activation of lysosomal enzymes, *Nature* **207** 1170–1173.

AMAR-COSTESEC, A., WIBO, M., THINES-SEMPOUX, D., BEAUFAY, H. & BERTHET, J. (1974) Analytical study of microsomes and isolated subcellular membranes from rat liver, *J. Cell Biol.* 62 717–745.

AMENDE, L. M. & DONLON, M. A. (1985) Isolation of cellular membranes from rat mast cells, *Biochim. Biophys. Acta* 812 713–720.

ANDERSON, R. L., MINTON, K. W., LI, G. C. & HAHN, G. M. (1981) Temperature induced homeoviscous adaptation of Chinese hamster ovary cells, *Biochim. Biophys. Acta* 641 334–348.

ANDERSON, R. L., TAO, T.-W. & HAHN, G. M. (1984) Cholesterol:phospholipid ratios decrease in heat resistant varients of B16 melanoma cells, in *Hyperthermic Oncology*, ed. Overgaard, J., Taylor & Francis, London, pp123–126.

ANDERSON, R. L., LUNEC, J. & CRESSWELL, S. R. (1985) Cholesterol content and heat sensitivity of nine mammalian cell lines, *Int. J. Hyperthermia* 1 337–347.

ANGHILERI, L. J. & DERMIETZEL, P. (1976) Cell coat in tumour cells:- effects of trypsin and EDTA: a biochemical and morphological study, *Oncology* 33 11–23.

ANGHILERI, L. J., MARCHA, C., ESCANYE, M. C. & ROBERT, C. M. J. (1985) Effects of extracellular calcium on calcium transort during hyperthermia of tumour cells, *Eur. J. Cancer Clin. Oncol.* 21 981–984.

ANTONIS, A. (1965) Semi-automated method for the colorimetric determination of plasma free fatty acids, *J. Lipid Res.* 6 307–312.

ASHWOOD-SMITH, M. J. (1985) Genetic damage is not produced by normal cryopreservation procedures involving either glycerol or DMSO. A cautionary note, however, on possible effects of DMSO, *Cryobiology* 22 427–433.

ATHREYA, B. H., GRIMES, E. L., LEHR, H. B., GREENE, A. & CORIELL, L. (1969) Differential susceptibility of epithelial cells and fibroblasts of human skin to freeze injury, *Cryobiology* 5 262–269.

ATKINSON, A., GATENBY, A. D. & LOWE, A. G. (1973) The determination of inorganic orthophosphate in biological systems, *Biochim. Biophys. Acta* 320 195–204.

AUGER, M., JARRELL, H. C. & SMITH, I. C. P. (1988) Interactions of the local anaesthetic tetracaine with membranes containing phosphatidylcholine and cholesterol: a ^2H-NMR study, *Biochem.* 27 4660–4667.

AUERSPERG, N. (1966) Differential heat sensitivity of cells in tissue culture, *Nature* 209 415–416.

BAKER, L. E. (1933) The cultivation of monocytes in fluid medium, *J. Exp.*

Med. 58 575.

BARKER, C. J. (1985) A study of the potential use of membrane perturbants in enhancing the hyperthermic treatment of cancer, Ph.D. Thesis, Durham University.

BASS, H., MOORE, J. A. & COAKLEY, W. T. (1978) Lethality in mammalian cells due to hyperthermia under oxic and hypoxic conditions, *Int. J. Radiat. Biol.* 33 57–62.

BASS, H., COAKLEY, W. T., MOORE, J. L. & TILLEY, D. (1982) Hyperthermia induced changes in the morphology of CHO-K_1 and their refractive inclusions, *J. Therm. Bio.* 7 231–242.

BATES, D. A. & MACKILLOP, W. J. (1985) The effect of hyperthermia on the sodium-potassium pump in Chinese hamster ovary cells, *Radiat. Res.* **103** 441–451.

BATES, D. A. & MACKILLOP, W. J. (1987) The effect of hyperthermia on intracellular K^+ in Chinese hamster ovary cells, *Cancer Lett.* 37 181–187.

BAUER, K. D. & HENLE, K. J. (1979) Arrtenius analysis of heat survival curves from normal and thermotolerant CHO cells, *Radiat. Res.* 78 251–263.

BEAUFAY, H., AMAR-COSTESEC, A., FEYTMANS, E., THINES - SEMPOUX, D., WIBO, M., ROBBI, M. & BERTHET, J. (1974) Analytical study of microsomes and isolated subcellular membranes from rat liver I. Biochemical methods, *J. Cell Biol.* **61** 188–200.

BELL, M. L., LAZARUS, H. M., HERMAN, A. H., EGDAHL, R. H. & RUTENBURG, A. M. (1971) *pH* dependent changes in cell membrane stability, *Proc. Soc. Exp. Biol. Med.* **136** 298–299.

BELSHAM, G. J., DENTON, R. M. & TANNER, M. J. A. (1980) Use of a novel rapid preparation of fat-cell plasma membranes employing Percoll to investigate the effects of insulin and adrenalin on membrane protein phosphonylation within intact fat cells, *Biochem. J.* **192** 457–467.

BHUYAN, B. H. (1979) Kinetics of cell kill by hyperthermia, *Cancer Res.* **39** 2277–2284.

BILLIG, D., CLARK, J. M., EWELL, A. J., CARTER, C. M. & GEBB, C. (1984) The separation of harvested cells from microcarriers: a comparison of methods, *Dev. Biol. Stand.* 55 67–75.

BLAUSTEIN, M. P. & GOLDMAN, D. E. (1966) Action of anionic and cationic nerve-blocking agents: experiment and interpretation, *Science* **153** 429–432.

BLIGH, E. G. & DYER, W. J. (1959) A rapid method of total lipid extraction and purification, *Can. J. Biochem. Physiol.* 37 911–917.

BLOOM, N. & FAWCETT, D. W. (1962) *A Textbook of Histology*, W. B. Saunders company, Philadelphia.

BODDIE, A. W. Jnr., WRIGHT, K., FAZER, J. W., STEPHENS, L. C., McBRIDE, C. M., WALLACE, S. & MARTIN, R. G. (1985) Selective occlusion and focal hyperthermia therapy of V-2 tumours in the rabbit hind limb, *Invest. Radiol.* 20 736–741.

BOONE, C. W., FORD, L. E., BOND, H. E., STUART, D. C. & LORENZ, D. (1969) Isolation of plasma membrane fragments from HeLa cells, *J. Cell Biol.* 41 378–392.

BOONSTRA, J., SCHAMHART, D. H. J., De LAAT, S. W. & Van WIJK, R. (1984) Analysis of K^+ and Na^+ transport and intracellular contents during and after heat shock and their role in protein synthesis in rat hepatoma cells, *Cancer Res.* 44 955–960.

BORRELLI, M. J., WONG, R. S. L. & DEWEY, W. C. (1986) A direct correlation between hyperthermia induced membrane blebbing and survival in synchronous G_1 CHO cells, *J. Cell Physiol.* 126 181–190.

BOSMANN, H. B., HAGOPIAN, A. & EYLAR, E. H. (1968) Cellular membranes: the isolation and characterization of the plasma and smooth membranes of HeLa cells, *Arch. Biochem. Biophys.* 128 51–69.

BOULANGER, Y., SCHREIER, S. & SMITH, I. C. P. (1981) Molecular details of anaesthetic-lipid interaction as seen by deuterium and phosphorous-31 nuclear magnetic resonance, *Biochem.* 20 6824–6830.

BOWLER, K., DUNCAN, D. J., GLADNELL, R. T. & DAVIDSON, T. F. (1973) Cellular heat injury, *Comp. Biochem. Physiol.* 45 441–449.

BOWLER, K. (1981) Heat death and cellular injury, *J. Therm. Bio.* 6 171–179.

BOWLER, K. (1987) Cellular heat injury: are membranes involved? In *Temperature and Animal Cells*, SEB symposium XLI, eds. Bowler, K. & Fuller, B. J., Cambridge:The Company of Biologists Ltd.

BOWLER, K., KASHMEERY, A. M. S. & BARKER, C. J. (1987) Heat sensitivity of tumour tissue, Poster Communication, in *Temperature and Animal Cells*, SEB symposium XLI, eds. Bowler, K. & Fuller, B. J., Cambridge:The Company of Biologists Ltd.

BRADFORD, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein-dye binding, *Analyt. Biochem.* 72 248–254.

BREASTED, J. H. (1930) *The Edwin Smith Surgical Papyrus*, University of Chicago Press, Chicago.

BRIDGEN, J., SNARY, D., CRUMPTON, M. J., BARNSTABLE, C., GOODFELLOW, P. & BODMER, W. F. (1976) Isolation and N-terminal amino acid sequence of membrane-bound human HLA-A and HLA-B antigens, *Nature* 261 200–205.

BRINKLEY, B. R., FISTEL, S. H., MARCUM, J. M. & PARDUE, R. L. (1980) Microtubules in cultured cells; indirect immunofluorescent staining with tubulin antibody, *Int. Rev. Cytol.* 63 59–95.

BRIVIO-HAUGLAND, R. P., LOUIS, S. L., MUSCH, K., WALDECK, N. & WILLIAMS, M. A. (1976) Liver plasma membranes from essential fatty acid deficient rats. Isolation, fatty acid composition and activities of 5′nucleotidase, ATPase and adenylate cyclase, *Biochim. Biophys. Acta* 433 150–163.

BROSSA, O., GARCIA, R., CAMUOT, R. A., GANTERO, B. & FEU, F. E. (1980) Cholesterol and phospholipid contents of the inner and outer membranes of highly purified mitochondria from Yoshida hepatoma AH-130, *I.R.C.S. Med. Sci. Alimentary Sysytem, Biochem., Cancer: Cell and Memb. Biol., Path.* 8 73–74.

BULL, J. M. (1984) A review of systemic hyperthermia, *Front. Radiat. Ther. Oncol.* 18 171–176.

BURDON, R. & CUTMORE, C. M. M. (1982) Human heat shock gene expression and the modulation of plasma membrane Na^+/K^+ATPase activity, *FEBS Lett.* 140 45–48.

BURDON, R. H., SLATER, A., McMATTON, M. & CATO, A. C. B. (1982) Hyperthermia and the heat shock proteins of HeLa cells, *Br. J. Cancer* 45 953.

BURDON, R. KERR, S. M., CUTMORE, C. M. M., MUNRO, J. & GILL, V. (1984) Hyperthermic Na^+/K^+ATPase and lactic acid production in some human tumour cells, *Br. J. Cancer* 49 437–445.

BURDON, R. H. (1985) *Hyperthermic Oncology*, ed. Overgaard, J., London, Taylor & Francis, pp223–230.

BURNS, C. P., LUTTENEGGER, D. G., DUDLEY, D. T., BUETTNER, G. R. & SPECTOR, A. A. & (1979) Effect of modification of plasma membrane fatty acid composition on fluidity and methotrexate transport in L1210 murine leukemia cells, *Cancer Res.* 39 1726–1732.

BURGER, F. J. & FUHRMAN, F. A. (1964) Evidence of injury by heat in mammalian tissues, *Am. J. Physiol.* 206 1057–1061.

BURNS, C. P., ROSENBERGER, J. A. & LUTTONEGGER, D. G. (1983) Selectivity in modification of the fatty acid composition of normal mouse tissues and membranes in vivo, *Ann. Nutr. Metab.* 27 268–277.

BURNS, C. P., LAMBERT, B. J., HAUGSTAD, B. N. & GUFFY, M. M. (1986)

Influence of rate of heating on thermosensitivity of L1210 leukemia cells: membrane lipids and M_r 70 000 heat shock protein, *Cancer Res.* 46 1882–1887.

BUSCH, W. (1866) Uber den einfluss welchen leftigere erysiplen zuweilen auf organisterte neubildungen ausuben, *Verh. Natur. Preuss Rhein Westphal.* 23 28–30.

BUTLER, K. W., SCHNEIDER, H. & SMITH, I. C. P. (1973) The effect of local anaesthetics on lipid multilayers, *Arch. Biochem. Biophys.* 154 548–554.

BYERS, M. R., FINK, B. R., KENNEDY, R. D., MIDDAUGH, M. E. & HENDRICKSON, A. E. (1973) Effects of lidocaine on axonal morphology, microtubules, and rapid transport in rabbit vagus nerve in vitro, *J. Neurol.* 4 125–143.

CALDERWOOD, S. K. (1987) Role of energy in cellular responses to heat, in *Temperature and Animal Cells*, SEB symposium XLI, eds. Bowler, K. & Fuller, B. J., Cambridge, the Company of Biologists Ltd.

CAMERON, E. & PAULING, L. (1979) *Cancer and Vitamin C*, W. W. Norton & Co., New York.

CAPCO, D. G., WAN, K. M. & PENMAN, S. (1982) The nuclear matrix: 3-dimensional architecture and protein composition, *Cell* 29 847–858.

CARMICHAEL, J., DeGRAFF, W. G., GAZDAR, A. F., MINNA, J. D. & MITCHELL, J. B. (1987) Evaluation of a tetrazolium-based semiautomated colorimetric assay: assessment of chemosensitivity testing, *Cancer Res.* 47 936–942.

CARROLL, P. M. & SEREDA, D. D. (1968) Cell membrane of uterine smooth muscle, *Nature* 217 666.

CHABANEL, A., FLAMM, M., SUNG, K. L. P., LEE, M. M., SCHATER, D. & CHIEN, S. (1983) Influence of cholesterol content on red cell membrane viscoelasticity and fluidity, *Biophys. J.* 44 171–76.

CHAKRABORTY, J. & Von STEIN, G. A. (1986) Pleomorphism of human prostatic cancer cells (DU145) in culture: the role of the cytoskeleton, *Exp. Mol. Pathol.* 44 235–245.

CHAKRAVARTHY, B. R., SPENCE, M. W., CLARKE, J. T. R. & COOK, H. W. (1985) Rapid isolation of neuroblastoma plasma membranes on Percoll gradients. Characterisation and lipid composition, *Biochim. Biophys. Acta* 812 223–233.

CHAM, B. E. & KNOWLES, B. R. (1976) A solvent system for delipidation of plasma or serum without protein precipitation, *J. Lipid Res.* 17 176–181.

CHAN, D. S. & WANG, H. H. (1984) Local anaesthetics can interact electrostatically with membrane proteins, *Biochim. Biophys. Acta* 770 55–64.

CHANG, H., SACCOMANI, G., RABON, E., SCHACKMANN, R. & SACHS,

G. (1977) Proton transport by gastric membrane vesicles, *Biochim. Biophys. Acta* 464 313–327.

CHANG, K. J., BENNETT, V. & CUATRECASAS, P. (1975) Membrane receptors as general markers for plasma membrane isolation procedures. The use of ^{125}I-labelled wheat germ agglutinin, insulin and cholera toxin, *J. Biol. Chem.* 250 488–500.

CHAPMAN, D. & WALLACH, D. H. (1968) The role of cholesterol in malignancy, *Prog. Exp. Tumour Res.* 22 275–316.

CHEN, S. S. (1974) Studies of radiocalcium efflux in single barnacle muscle fibres: effects of procaine and external divalent cations, *J. Physiol.* 238 313–328.

CHENG, K.-H., HUI, S. W. & LEPOCK, J. R. (1987) Protection of the membrane calcium adenosine triphosphate by cholesterol from thermal inactivation, *Cancer Res.* 47 1255–1262.

CHU, E. H. Y., SANFORD, K. K. & EARLE, W. R. (1958) Comparative chromosomal studies on mammalian cells in culture. II. Mouse sarcoma producing cell strains and their derivations, *J. Natl. Cancer Inst.* 21 729.

CHU, G. L. & DEWEY, W. C. (1987) Effect of hyperthermia on intracellular *pH* in Chinese hamster ovary cells, *Radiat. Res.* 110 439–449.

CLARK, J. M. & HIRTENSTEIN M. D. (1980) Optimal conditions for the culture of animal cells on microcarriers, *In Vitro* 16 227.

COLEY, W. (1893) The treatment of malignant tumours by repeated inoculations of erysipelas: with a report of ten original cases, *Am. J. Med. Sci.* 105 487–511.

COLLARD, J. G. & De WILDT, A. (1978) Localization of the lipid probe 1,6-diphenyl-1,3,5 hexatriene (DPH) in intact cells by fluorescence microscopy, *Exp. Cell Res.* 116 447–450.

COLLEY, C. M. & METCALFE, J. C. (1972) The localisation of small molecules in lipid bilyers, *FEBS Lett.* 24 241–246.

COLLIER, N. C. & SCHLESINGER, M. J. (1986) The dynamic state of heat shock proteins in chicken embryo fibroblasts, *J. Cell Biol.* 103 1495–1507.

CONSTANTINESCU, A., BAJENARU, FRANGOPOL, P.T. & MARGINEANU, D. G. (1986) Does procaine really fluidise the erythrocyte membrane? *Rev. Roum. Biochim.* 23 97–102.

COOPER, P. D., WILSON, J. N. & BURT, A. M. (1959) The bulk growth of animal cells in continuous suspension culture, *J. Gen. Micro.* 21 702–720.

COOPER, P. H. & STANWORTH, D. R. (1976) Characterisation of calcium-activated adenosine trisphosphatase in the plasma membrane of rat mast cells,

Biochem. J. 156 691–700.

CORIELL, L. L. (1979) Presentation, storage and shipment, *Cell culture, section 1: Basic methods in enzymology*, eds. Jakoby, W. B. & Pastan, I. H. Vol. LVIII pp29–36.

COSS, R. A. & DEWEY, W. C. (1988) Heat sensitisation of G_1 and S phase cells by procaine *HCl*. II: toxicity and probability of dividing following treatment, *Int. J. Hyperthermia* 4 687–697.

COSSINS, A. R. (1976) Changes in muscle lipid composition and resistance adaptation to temperature in the freshwater crayfish, Austrop-otamobius pallipes, *Lipids* 11 307–316.

COSSINS, A. R., FRIEDLANDER, M. J. & PROSSER, C. L. (1977) Correlations between behavioural temperature adaptations of goldfish and the viscosity and fatty acid composition of their synaptic membranes, *J. Comp. Physiol.* 120 109–121.

COSSINS, A. R. & PROSSER, C. L. (1978) Evolutionary adaptation of membranes to temperature, *Proc. Natn. Acad. Sci. (USA)* 75 2040–2043.

COSSINS, A. R., BOWLER, K. & PROSSER, C. L. (1981) Homeoviscous adaptation and its effect upon membrane-bound proteins, *J. Therm. Bio.* 6 183–187.

COSSINS, A. R. & SINENSKY, M. (1984) Adaptation of membranes to temperature, pressure and exogeneous lipids, in *Physiology of Membrane Fuidity*, Vol. II, ed. Shinitzky, M., Boca Raton, USA CRC Press, pp1–20.

COSSINS, A. R., BEHAN, M., JONES, G. & BOWLER, K. (1986) Lipid-protein interactions in the adaptive regulation of membrane function, *Bioch. Soc. T.* 15 77–81.

COSSINS, A. R. & BOWLER, K. (1987) *Temperature Biology of Animals*, Chapman & Hall, London.

COURCHAINE, A. J., MILLER, W. H. & STEIN, D. B. Jnr. (1959) Rapid semimicro procedure for estimating free and total cholesterol, *Clin. Chem.* 5 609–614.

CRESS, A. E. & GERNER, E. W. (1980) Cholesterol levels inversely reflect the thermal sensitivty of mammalian cells in culture, *Nature* 283 610–617.

CRESS, A. E., CULVER, P. S., MOON, T. E. & GERNER, E. W. (1982) Correlation between amounts of cellular membrane components and sensitivity to hyperthermia in a variety of mammalian cell lines in culture, *Cancer Res.* 42 1716–1721.

CULVER, P. S. & GERNER, E. W. (1982) Temperature acclimation and specific cellular components in the regulation of thermal sensitivity of mammalian cells,

Natl. Cancer Inst. Monogr. 61 99–101.

De DUVE, C., BERTHET, J., HERS, H. G. & DUPRET, L. (1949) Le systeme hexose-phosphatasique. I. Existence d'une glucose-6-phosphatase spécifique dans le foie, *Bull. Soc. Chim. Biol.* 31 1242–1253.

De DUVE, C. (1964) Principles of tissue fractionation, *J. Theor. Biol.* 6 33–59.

De PIERRE, J. W. & KARNOVSKY, M. L. (1973) Plasma membranes of mammalian cells (review). A review of methods for their characterisation and isolation, *J. Cell Biol.* 56 275–303.

DENIZOT, F. & LANG, R. (1986) Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliabilty, *J. Immunol. Methods* 89 271–277.

DENNING, G. M., FIGARD, P. H. & SPECTOR, A. A. (1982) Effect of fatty acid modification on prostaglandin production by cultured 3T3 cells, *J. Lipid Res.* 23 584–596.

DENNIS, W. H. & YATVIN, M. B. (1981) Correlation of hyperthermic sensitivity and membrane microviscosity in E.Coli K1060, *Int. J. Radiat. Biol.* 39 265–271.

DEWEY, W. C., HOPWOOD, L. E., SAPARETO, S. A. & GERWECK, L. E. (1977) Cellular responses to combinations of hyperthermia and radiation, *Radiol.* 123 463–474.

DICKENS, B. F. & THOMPSON, G. A. (1982) Phospholipid molecular species alterations in microsomal membranes as an initial key step during cellular acclimation to low temperature, *Biochem.* 21 3604–3611.

DICKSON, J. A. & OSWALD, B. E. (1976) The sensitivity of a malignant cell line to hyperthermia ($42°C$) at low intracellular pH, *Br. J. Cancer* 34 262–271.

DICKSON, J. A. & SUZANGER, M. (1976) A predictive *in vitro* assay for the sensitivity of human solid tumours to hyperthermia ($42°C$) and its value in patient management, *Clin. Oncol.* 2 141–155.

DIPPLE, I., GORDON, L. M. & HOUSLAY, M. D. (1982) The activity of 5′nucleotidase in liver plasma membranes is affected by the increase in bilayer fluidity acheived by anionic but not by cationic drugs, *J. Biol. Chem.* 257 1811–1815.

DOI, O., DOI, F., SCHROEDER, F., ALBERTS, A. W. & VAGELOS, P. R. (1978) Manipulation of fatty acid composition of membrane plasma and its effects on cell growth in mouse LM cells, *Biochim. Biophys. Acta* 509 239–250.

DRAYE, J. P., COURTOY, P. J., QUINTART, J. & BAUDHUIN, P. (1987) Relations between plasma membrane and lysosomal membrane. 2. Quantitative evaluation of plasma membrane marker enzymes in the lysosomes, *Eur. J.*

Biochem. 170 405–411.

DULBECCO, R. & VOGT, M. (1954) Plaque formation and isolation of pure lines with poliomyelitis viruses, *J. Exp. Med.* 99 167–182.

DUNBAR, L. M. & BAILEY, J. M. (1975) Enzyme deletions and essential fatty acid metabolism in cultured cells, *J. Biol. Chem.* 250 1152–1153.

DURAND, R. E. (1978) Potentiation of radiation lethality by hyperthermia in a tumour model: effects of sequence, degree and duration of heating, *Int. J. Radiat. Oncol. Biol. Phys.* 4 401–406.

EAGLE, H. (1959) Amino acid metabolism in mammalian cell cultures, *Science* 130 432–437.

EDSTROM, A., ERKELL, L. J. & HANSSON, H.-A. (1975) Reversal of morphological differentiation of mouse neuroblastoma cells by mitosis-inhibitors and anaesthetics, *Virchows Arch. B. Cell Pathol.* 19 101–113.

EDWARDS-WEBB, J. D. & GURR, M. I. (1988) The influence of dietary fats on the chemical composition and physical properties of biological membranes, *Nutr. Res.* 8 1297–1305.

ELSDALE, T. & BARD, J. (1972) Collagen substrata for studies on cell behaviour, *J. Cell Biol.* 54 626–637.

EMMELOT, P. & BOS, C. J. (1966) Studies on plasma membranes III. Mg^{2+}-ATPase, $(Na^{+} - K^{+} - Mg^{2+})$-ATPase and 5′nucleotidase activity of plasma membranes isolated from rat liver, *Biochim. Biophys. Acta* 120 369–382.

EMMELOT, P. & BOS, C. J. (1971) Studies on plasma membranes XIV Adenyl cyclase in plasma membranes isolated from rat and mouse livers and hepatomas, and its hormone sensitivity, *Biochim. Biophys. Acta* 249 285–292.

EMMELOT, P., BOS, C. J., Van HOEVEN, R. P. & Van BLITTERSWIJK, W. J. (1974) Isolation of plasma membranes from rat and mouse livers and hepatomas, in *Methods in Enzymology*, eds. Fleischer, S. & Packer, L., Acedemic Press, London, Vol. XXXI pp75–90.

ESSER, A. F. & SOUZA, K. A. (1974) Correlation between thermal death and membrane fluidity in Bacillus stearothermophilus, *Proc. Natn. Acad. Sci. (USA)* 71 4111–4115.

EVANS, W. H. (1970) Fractionation of liver plasma membranes prepared by zonal centrifugation, *Biochem. J.* 116 833–842.

EVANS, W. H. (1982) Subcellular membranes and isolated organelles: preparative techniques and criteria for purity, in *Technologies in Lipid and Membrane Biochemistry*, Elsvier scientific publishers, North-Holland, B407a pp1–46.

EWELL, A. J. & CARTER, C. M. (1982) Harvesting cells from microcarriers,

In Vitro 18 312–313.

FAJARDO, L. F., EGBERT, B., MARMOR, J. & HAHN, G. M. (1980) Effects of hyperthermia in a malignant tumour, *Cancer* 45 613–623.

FERGUSON, K. A., GLASER, M., BAYER, W. H. & VAGELOS, P. R. (1975) Alteration of fatty acid composition of LM cells by lipid supplementation and temperature, *Biochem.* 14 146–151.

FERNANDEZ, M. S. (1980) Formation of micelles and membrane action of the local anaesthetic tetracaine hydrochloride, *Biochim. Biophys. Acta* 597 83–91.

FEY, E. G. & PENMAN, S. (1984) Tumour promoters induce a specific morphological signature in the nuclear matrix-intermediate filament scaffold of Madin-Darby canine kidney (MDCK) cell colonies, *Proc. Natn. Acad. Sci. (USA)* 81 4409–4413.

FEY, E. G., WAN, K. M. & PENMAN, S. (1984) Epithelial cytoskeletal framework and nuclear matrix intermediate filament scaffold: three dimensional organisation and protein composition, *J. Cell Biol.* 98 1973–1984.

FIELD, S. B. (1987a) Studies relevant to a means of quantifying the effects of hyperthermia, *Int. J. Hyperthermia* 3 291–296.

FIELD, S. B. (1987b) Hyperthermia in the treatment of cancer, *Phys. Med. Biol.* 32 789–811.

FISHER, G. A., LI, G. C. & HAHN, G. M. (1982) Modification of the thermal response by D_2O. I. Cell survival and the temperature shift, *Radiat. Res.* 92 530–540.

FORBUSH, B. (1983) Assay of $Na;K$-ATPase in plasma membrane preparations: increasing the permeability of membrane vesicles using sodium dodecyl sulphate buffered with bovine serum albumin, *Analyt. Biochem.* 128 159–163.

FRANCE, W. W., SCHMID, E., KARTENBECK, J., MAYER. D., HACKER, H. J., BANNASH, P., OSBORN, M., WEBER, K., DENK, H., WANSON, J. C. & DROCHMANS, P. (1979) Characterisation of the intermediate sized filaments in liver cells by immunofluorescence and electron microscopy, *Biol. Cellulaire* 34 99–110.

FRANKE, W. W., SCHMID, E., KARTENBECK, J., MAYER, D., HACKER, H.-J., BANNASH, P., OSBORN, M., WEBER, K., DENK, H., WANSON, J.-C. & DROCHMANS, P. (1979) Characterisation of the intermediate-sized filaments in liver cells by immunofluorescence and electron microscopy, *Biol. Cellulaire* 34 99–110.

FRENCH, S. W., KONDO, I., IRIE, T., IHRIG, T. J., BENSON, N. & MUNN, R. (1982) Morphologic study of intermediate filaments in rat hepatocytes, *Hepatology* 2 29–38.

FRIEND, C., PATULEIA, M. C. & NELSON, J. B. (1966) Antibiotic effect of tylosin on a mycoplasma contaminant in a tissue culture leukemia cell line, *Proc. Soc. Exp. Biol. Med.* 121 1009.

FUKUZAWA, K., CHIDA, H., TOKUMURA, A. & TSUKATANI, H. (1981) Antioxidative effect of α-tocopherol incorporation into lezithin liposomes on ascorbic acid-Fe^{2+}-induced lipid peroxidation, *Arch. Biochem. Biophys.* 206 173–180.

GASPAR, G., ALANIZ, M. J. T. de. & BRENNER, R. R. (1975) Uptake and metabolism of exogenous Eicosa-8,11,14-trienoic acid in minimal deviation hepatoma 7288C cells, *Lipids* 10 726–731.

GASPAR, G., ALANIZ, M. J. T. de. & BRENNER, R. R. (1977) Incorporation and metabolism of stearic, oleic, linoleic and α-linoleic acids in minimal deviation hepatoma 7288C cells, *Mol. Cell. Biochem.* 16 197–203.

GEBB, C. L., LUNDGREN, B., CLARK, J. M., LINDSKOG, U. (1982) Harvesting and subculturing cells growing on denatured-collagen coated microcarriers (Cytodex 3), *Dev. Biol. Stand.* 55.

GENNA, J. M., COFFE, G. & PUDLES, J. (1980) Effects of tertiary amine local anaesthetics on the assembly and disassembly of brain microtubules in vitro, *Eur. J. Biochem.* 110 457–464.

GEORGE, A. M., LUNEC, J. & CRAMP, W. A. (1983) Effect of membrane fatty acid changes on the radiation sensitivity of human lymphoid cells, *Int. J. Radiat. Biol.* 43 363–378.

GERNER, E. W., CONNOR, W. G., BOONE, M. L. M., DOSS, J. D., MAYER, E. G. & MILLER, R. G. (1975) The potential of localised heating as an adjunct to radiation therapy, *Radiol.* 116 433–439.

GERNER, E. W., CRESS, A. E., STICKNEY, D. G., HOLMES, D. K. & CULVER, P. (1980) Factors regulating membrane permeability alter thermal resistance, *Ann. N. Y. Acad. Sci.* 335 215–233.

GERWECK, L. E. (1977) Modification of cell lethality at elevated temperatures: the *pH* effect, *Radiat. Res.* 70 224–235.

GERWECK, L. E., NYGAARD, T. G. & BURLETT, M. (1979) Response of cells to hyperthermia under acute and chronic hypoxic conditions, *Cancer Res.* 39 966–972.

GERWECK, L. E., DAHLBERG, W. C., EPSTEIN, L. & SHIMM, D. S. (1984) Influence of nutrient and energy deprivation on cellular response to single and fractionated heat treatments, *Radiat. Res.* 99 573–581.

GERWECK, L. E. (1988) Modifiers of thermal effects: environmental factors, *Hyperthermia Oncol.* 1 83–98.

GEYER, R. P., BENNET, A. & ROHR, A. (1962) Fatty acids of the triglycerides and phospholipids of HeLa cells and strain L fibroblasts, *J. Lipid Res.* 3 80–83.

GEYER, R. P. (1967) *Lipid metabolism in tissue culture cells*, eds. Rothblat, G. H. & Kritchevsky, Y. D., Wistar Institute Press, Philadelphia, pp33–44.

GILMAN, A. G. (1970) A protein binding assay for adenosine 3′5′-cyclic monophosphate, *Proc. Natn. Acad. Sci. (USA)* 67 305–312.

GILMORE, R., COHN, N. & GLASER, M. (1979) Fluidity of LM cell membranes with modified lipid compositions as determined with 1,6-diphenyl-1,3,5-hexatiene, *Biochem.* 18 1042–1049.

GIOVANELLA, B. C., MORGAN, A. C., STEHLIN, J. S. & WILLIAMS, L. J. (1973) Selective lethal effect of supranormal temperatures on mouse sarcoma cells, *Cancer Res.* 33 2568–2578.

GIOVANELLA, B. C., STEHLIN, J. S. & MORGAN, A. C. (1976) Selective lethal effect of supranormal temperatures on human neoplastic cells, *Cancer Res.* 36 3944–3950.

GLASER, M., FERGUSON, K. A. & VAGELOS, P. R. (1974) Manipulation of the phospholipid composition of tissue culture cells, *Proc. Natn. Acad. Sci. (USA)* 71 4072–4076.

GLASS, J. R., DEWITT, R. G. & CRESS, A. E. (1985) Rapid loss of stress fibres in Chinese hamster ovary cells, *Cancer Res.* 45 258–269.

GOLDMAN, R. D., GOLDMAN, A. E., GREEN, K. J., JONES, J. C. R., JONES, S. M. & YANG, H.-Y. (1986) Intermediate filament networks: organisation and possible functions of a diverse group of cytoskeletal elements, *J. Cell Sci.* 5 69–97.

GOLDSTEIN, D. B. (1984) The effects of drugs on membrane fluidity, *Ann. Rev. Pharm. Toxicol.* 24 43–64.

GONZALEZ-MENDEZ, R., MINTON, K. W. & HAHN, G. M. (1982) Lack of correlation between membrane lipid composition and thermotolerance in Chinese hamster ovary cells, *Biochim. Biophys. Acta* 692 168–170.

GORDON, L., WHETTON, A. D., RAWAL, S., ESGATE, J. A. & HOUSLAY, M. D. (1983) Pertubation of liver plasma membranes induced by Ca^{2+} are detected using a fatty acid spin label and adenylate cyclase as membrane probes, *Biochim. Biophys. Acta* 729 104–114.

GOTLIB, L. J. & SEARLS, D. B. (1980) Plasma membrane isolation on DEAE-sephadex beads, *Biochim. Biophys. Acta* 602 207–212.

GOTLIB, L. J. (1982) Isolation of cell plasma membranes on microcarrier culture beads, *Biochim. Biophys. Acta* 685 21–26.

GOTO, I., KOBAYASHI, T., ANTOKU, Y., TOBIMATSU, S. & KUROIWA, Y. (1986) Adrenoleukodystrophy and varients: Clinical, neurophysiological and biochemical studies in patients and family members, *J. Neurol. Sci.* 72 103–112.

GREEN, K. J. & GOLDMAN, R. D. (1986) Evidence for an interaction between the cell surface and intermediate filaments in cultured fibroblasts, *Cell Motil. Cytoskeleton* 6 389–405.

GREEN, L. M., READE, J. L. & WARE, C. F. (1984) Rapid colorimetric assay for cell viability: application to the quantitation of cytotoxic and growth inhibitory lymphokines, *J. Immunol. Methods* 70 257–268.

GREENBERG, M. & TSONG, T. Y. (1984) Detergent solubilisation and affinity purification of a local anaesthetic binding protein from mammalian axonal membranes, *J. Biol. Chem.* 259 13241–13245.

GREENGARD, O., FEDERMAN, M. & KNOX, W. E. (1972) Cytomorphometry of developing rat liver and its application to enzymic differentiation, *J. Cell Biol.* 52 261–272.

GUFFY, M. M., ROSENBERGER, J. A., SIMON, I. & BURNS, C. P. (1982) Effect of cellular fatty acid alteration on hyperthermic sensitivity in cultured L1210 mouse leukemia cells, *Cancer Res.* 42 3625–3630.

HAEFFNER, E. W., KOLBE, K., SCHROETER, D. & PAWELETZ, N. (1980) Plasma membrane heterogeneity in ascites tumour cells. Isolation of a light and a heavy membrane fraction of the glycogen-free Ehrlich-Lettre substratum, *Biochim. Biophys. Acta* 603 36–51.

HAHN, G. M. (1974) Metabolic aspects of the role of hyperthermia in mammalian cell inactivation and their possible relevance to cancer treatment, *Cancer Res.* 34 3117–3123.

HAHN, G. M., BRAUN, J. & HAR-KEDAR, I. (1975) Thermochemotherapy: synergism between hyperthermia (42-43°C) and adriamycin (or bleomycin) in mammalian cell inactivation, *Proc. Natn. Acad. Sci. (USA)* 72 937–940.

HAHN, G. M. & STRANDE, D. P. (1976) Cytoxic effects of hyperthermia and adriamycin on Chinese hamster cells, *J. Natl. Cancer Inst.* 57 1063–1067.

HAHN, G. M., LI, G. C. & SHIU, E. (1977) Interaction of amphotericin B and 43°C hyperthermia, *Cancer Res.* 37 761–764.

HAHN, G. M. (1980) Comparison of malignant potential of $10T\frac{1}{2}$ cells and transformants with their survival responses to hyperthermia and to amphotericin B, *Cancer Res.* 40 3763–3767.

HAHN, G. M. (1982) *Hyperthermia and Cancer*, Plenum, New York.

HARISIADIS, L., HALL, E. J., KRAIJEVIC, U. & BOREK, C. (1975) Hyper-

thermia: biological studies at the cellular level, *Radiol.* 117 447–452.

HARRIS, J. R., MURTHY, A. K. & BELLI, J. A. (1977) Repair following combined X-ray and heat at 41°C in plateau-phase mammalian cells, *Cancer Res.* 37 3374.

HAZEL, J. R. & PROSSER, C. L. (1974) Molecular mechanisms of temperature compensation in poikilotherms, *Ann. R. Physl.* 54 620–677.

HAZEL, J. R. (1979) Influence of thermal acclimation on membrane lipid composition of rainbow trout liver, *Am. J. Physiol.* 236 R91–101.

HAZEL, J. R. (1984) Effects of temperature on the structure and metabolism of cell membranes of fish, *Am. J. Physiol.* 246 R460–470.

HEILBRUNN, L. V. (1924) The colloid chemistry of protoplasm. IV. The heat coagulaion of protoplasm, *Am. J. Physiol.* 69 190–199.

HEINE, U., SEVERAK, L., KONDRATICK, J. & BONAR, R. A. (1971) The behaviour of HeLa S_3 cells under the influence of supranormal temperatures, *J. Ultrastruc. Res.* 34 375–396.

HENLE, K. J. & LEEPER, D. B. (1976) Interaction of hyperthermia and radiation in CHO cells: recovery kinetics, *Radiat. Res.* 66 505–518.

HENLE, K. J. & DETHLEFSEN, L. A. (1978) Heat fractionation and thermotolerance: a review, *Cancer Res.* 38 1842–1851.

HENLE, K. J. & LEEPER, D. B. (1979) Effects of hyperthermia (45°C) on macromolecular synthesis in Chinese hamster ovary cells, *Cancer Res.* 39 2665–2674.

HENLE, K. J. & ROTI ROTI, J. L. (1988) Response of cultured mammalian cells to hyperthermia, *Hyperthermia Oncol.* 1 57–82.

HENNING, R. & HEIDRICH, H. G. (1974) Membrane lipids of rat liver lysosomes prepared by free-flow electrophoresis, *Biochim. Biophys. Acta* 345 326–335.

HIDVÉGI, E. J., YATVIN, M. B., DENNIS, W. H. & HIDVÉGI, E. (1980) Effect of altered membrane lipid composition and procaine on hyperthermic killing of ascites tumour cells, *Oncology* 37 360–363.

HIGGINS, P. J., DARZYNKIEWICZ, Z. & MELAMED, M. R. (1983) Secretion of albumin and alpha-foetoprotein by DMSO-stimulated hepatocellular carcinoma cells, *Br. J. Cancer* 48 485–493.

HIRTENSTEIN, M. & CLARK, J. M. (1980) *Tissue cuture in medical research*, eds. Richards, R. & Rajan, K., Pergamon Press, Oxford, pp97.

HIRTENSTEIN, M. D., CLARK, J. M. & GEBB, C. (1981) A comparison of

various laboratory scale culture configurations for microcarrier culture of animal cells, *Dev. Biol. Stand.* 50 73–80.

HOFER, K. G., BRIZZARD, B. & HOFER, M. G. (1979) Effect of lysosome modification on the heat potentiation of radiation damage and direct heat death of BP-8 sarcoma cells, *Eur. J. Cancer* 15 1449–1457.

HONN, K. V., SINGLEY, J. A. & CHAVIN, W. (1975) Fetal bovine serum: a multivariate standard (38805), *Proc. Soc. Exp. Biol. Med.* 149 344–347.

HORNG, C.-B. & McLIMANS, W. (1975) Primary suspension culture of calf anterior pituitary cells on a microcarrier surface, *Biotechnol. Bioeng.* 17 713–732.

HORWITZ, A. F., HATTEN, M. E. & BURGER, M. M. (1974) Membrane fatty acid replacements and their effect on growth and lectin-induced agglutinability, *Proc. Natn. Acad. Sci. (USA)* 71 3115–3119.

HORWITZ, A. F., WIGHT, A., LUDWIG, P. & CORNELL, R. (1978) Interrelated lipid alterations and their influence on the proliferation and fusion of cultured myogenic cells, *J. Cell Biol.* 77 334–357.

HUBBELL, W. L., METCALFE, J. C. METCALFE, S. M. & McCONNELL, H. M. (1970) The interaction of small molecules with spin-labelled erythrocyte membranes, *Biochim. Biophys. Acta* 219 415–427.

HUME, S. P. & FIELD, S. B. (1977) Acid phosphatase activity following hyperthermia of mouse spleen and its implication in heat potentiation of X-ray damage, *Radiat. Res.* 72 145–153.

HURWITZ, C. & TOLMACH, L. J. (1969) Time-lapse cinemicrographic studies of X-irradiated HeLa S3 cells. II. Cell fusion, *Biophys. J.* 9 1131–1143.

HYMAN, B. T. & SPECTOR, A. A. (1981) Accumulation of $n-3$ polyunsaturated fatty acids by cultured human Y79 retinoblastoma cells, *J. Neurochem.* 37 60–69.

INBAR, M. (1976) Fluidity of membrane lipids: a single cell analysis of mouse normal lymphocytes and malignant lymphoma cells, *FEBS Lett.* 67 180–185.

ISHII, M., MIYAZAKI, Y., OTSUKI, H. & GOTO, Y. (1985) The intermediate filaments in human hepatocytes, *Tohoku J. Exp. Med.* 147 317–329.

ISHIZAKA, T., HIRATA, F., STERK, A. R., ISHIZAKA, K. & AXELROD, J. A. (1981) Bridging of IgE receptors activates phospholipid methylation and adenylate cyclase in mast cell plasma membranes, *Proc. Natn. Acad. Sci. (USA)* 78 6812–6816.

JAIN, M. K. & WU, N. M. (1977) Effect of small molecules on the dipalmitoyl lecithin liposomal bilayer. III Phase transition in lipid bilayer, *J. Membr. Biol.*

34 157–201.

JARES, J. J. & WARREN, S. L. (1939) Physiological effects of radiation I. A study of the in vitro effect of high fever temperatures upon certain experimental animal tumours, *Am. J. Roentgenol.* 41 685.

JOHNSON, F. H., EYRING, H. & STORES, B. J. (1974) *The Theory of Rate Processes in Biology and Medicine*, New York, Wiley, pp253–261.

JOHNSON, H. A. (1974) On the thermodynamics of cell injury: some insights into the molecular mechanism, *Am. J. Pathol.* 75 13–25.

JOHNSON, S. M. (1979) A new specific cholesterol assay gives reduced cholesterol/phospholipid molar ratios in cell membranes, *Analyt. Biochem.* 95 344–350.

JOHNSON, S. M. (1981) Steady state diphenyl hexatriene fluororescence polarisation in the study of cells and of damage by heat and heat plus plasma membranes, in *Fluorescent Probes*, eds. Beddard, G. S. & West, M. A., Academic Press, pp143–158.

JOHNSTON, P. V. & ROOTS, B. I. (1964) Brain lipid fatty acids and temperature acclimation, *Comp. Biochem. Physiol.* 11 303–309.

JORRITSMA, J. M. B., KAMPINGA, H. H. & KONINGS, A. W. T. (1984) Role of DNA polymerases in the mechanisms of damage by heat and heat plus radiation in mammalian cells, *Hyperthermic Oncol.* 1, Summary papers edited by Overgaard, A., London & Philadelphia, pp61–64.

JUNG, H. (1986) A generalised concept for cell killing by heat, *Radiat. Res.* **106** 56–72.

KADUCE, T. L., SPECTOR, A. A. & BAR, R. S. (1982) Linoleic acid metabolism and prostaglandin production by cultured bovine pulmonary artery endothelial cells, *Arteriosclerosis* 2 380–389.

KAMPINGA, H. H., MULLENDERS, L. H. F. & KONINGS, A. W. T. (1988) Effect of hyperthermia on DNA loop size in HeLa S_3 cells, *Int. J. Radiat. Biol.* 53 291–300.

KAPISZEWSKA, M. & HOPWOOD, L. E. (1986) Changes in bleb formation following hyperthermia treatment of Chinese hamster ovary cells, *Radiat. Res.* 105 405–412.

KAPP, D. S. & HAHN, G. M. (1979) Thermosensitisation by sulfhydryl compounds of exponentially growing Chinese hamster cells, *Cancer Res.* 39 4630–4635.

KARNOVSKY, M. J. (1965) A formaldehyde-glutaraldehyde fixative of high osmolality for use in electron microscopy, *J. Cell Biol.* 27 137A.

KASHNIG, D. M. & KASPER, C. B. (1969) Isolation, morphology and compo-

sition of the nuclear membrane from rat liver, *J. Biol. Chem.* 244 3786–3792.

KELLEY, P. M. & SCHLESINGER, M. J. (1978) The effect of amino acid analogues and heat shock on gene expression in chicken embryo fibroblasts, *Cell* 15 1277–1286.

KELUSKY, E. C. & SMITH, I. C. P. (1983) Characterisation of the binding of the local anaesthetics procaine and tetracaine to model membranes of phosphatidylethanolamine: a deuterium nuclear magnetic resonance study, *Biochem.* 22 6011–6017.

KELUSKY, E. C., BOULANGER, Y., SCHREIER, S. & SMITH, I. C. P. (1986) A ^2H-NMR study on the interactions of the local anaesthetic tetracaine with membranes containing phosphatidylseline, *Biochim. Biophys. Acta* 856 85–90.

KIM, J. H., KIM, S. H., HAHN, E. W. & SONG, C. W. (1978) 5-Thio-D-glucose selectivity potentiates hyperthermic killing of hypoxic tumour cells, *Science* 200 206–207.

KIM, S. H., KIM, J. H. & HAHN, E. W. (1975) Enhanced killing of hypoxic tumour cells by hyperthermia, *Br. J. Radiol.* 48 872–874.

KIM, S. H., KIM, J. H., HAHN, E. W. & ENSIGN, N. A. (1980) Selective killing of glucose and oxygen-deprived HeLa cells by hyperthermia, *Cancer Res.* 40 3459–3462.

KIMELBERG, H. K. (1977) The influence of membrane fluidity on the activity of membrane bound enzymes, *Cell Surf. Rev.* 3 205–293.

KING, M. E., STAVENS, B. W. & SPECTOR, A. A. (1977) Diet induced changes in plasma membrane fatty acid composition affect physical properties detected with a spin-label probe, *Biochem.* 16 5280–5285.

KING, M. E. & SPECTOR, A. A. (1978) Effect of specific fatty acyl enrichments on membrane physical properties detected with a spin label probe, *J. Biol. Chem.* 253 6493–6501.

KINGSTON, C. A., LADHA, S., MANNING, R. & BOWLER, K. (1989) Hyperthermic cell death of HTC cells in culture: a comparison of assays, *Bioch. Soc. T.* 17 531–532.

KOGURT, M. (1980) Are these strategies of microbial adaptation to extreme environments? *Trends Biochem. Sci.* 5 15–18.

KOIZUMI, K., ITO, Y., KOJIMA, K. & FUIJII, T. (1976) Isolation and characterisation of the plasma membranes from rat hepatomas and from normal rat livers including newborn, regenerating and adult rat livers, *J. Biochem.* 79 739–748.

KOIZUMI, K., SHIMIZU, S., KOIZUMI, K. T., NISHIDA, K., SATO, C., OTA,

K. & YAMAWAKA, N. (1981) Rapid isolation and lipid characterisation of plasma membranes from normal and malignant lymphoid cells of mouse, *Biochim. Biophys. Acta* 649 393–403.

KONINGS, A. W. T. (1985) Development of thermotolerance in mouse fibroblast LM cells with modified membranes and after procaine treatment, *Cancer Res.* 45 2016–2019.

KONINGS, A. W. T. & RUIFROK, A. C. C. (1985) Role of membrane lipids and membrane fluidity in thermosensitivity and thermotolerance of mammalian cells, *Radiat. Res.* 102 86–98.

KURODA, Y. & FUJIWARA, Y. (1987) Locations and dynamical perturations for lipids of cationic forms of procaine, tetracaine and dibucaine in small unilamellar phosphotidylcholine vesicles as studied by nuclear overhauser effects in ^1H nuclear magnetic resonance spectroscopy, *Biochim. Biophys. Acta* 903 395–410.

KWOCK, L., LIN, P.-S., HEFTER, K. & WALLACH, D. F. H. (1978) Impairment of Na^+-dependent amino acid transport in cultured human T-cell line by hyperthermia and irradiation, *Cancer Res.* 38 83–87.

LAI, C.-S., HOPWOOD, L. E. & SWARTZ, H. M. (1980) ESR studies on membrane fluidity of Chinese hamster ovary cells grown on microcarriers and in suspension, *Exp. Cell Res.* 130 437–442.

LANDS, W. E. M. & CRAWFORD, C. G. (1976) Enzymes of membrane phospholipid metabolism in animals, in *The Enzymes of Biological Membranes*, Vol. II, ed. Martonosi, A., Plenum Press, New York, p3.

LANDS, W. E. M. & HART, P. (1966) The control of fatty acid composition in glycerolipids, *J. Amer. Oil Chem. Soc.* 43 290–295.

LAVAL, F. & MICHEL, S. (1982) Enhancement of hyperthermia-induced cytotoxicity upon ATP deprivation, *Cancer Lett.* 15 61–65.

LAZARIDES, E. (1980) Intermediate filaments as mechanical integrators of cellular space, *Nature* 283 249–256.

LEE, A. G. (1982) Membrane studies using fluorescence spectroscopy, in *Techniques in the Life Sciences, Lipid and Membrane Biochemistry*, Elsevier scientific publishers, Ireland, *New. Pythol.* B422 pp1–49.

LEE, A. G. (1985) Symposium on nutritional aspects of membrane structure and function. Some principles of membrane structure, *Proc. Nutr. Soc.* 44 147–156.

LEE, D. C. & CHAPMAN, D. (1987) The effects of temperature on biological membranes and their models, in *Temperature and Animal Cells*, S.E.B. symposium XLI, eds. Bowler, K. & Fuller, B. J., Cambridge: The Company of Biologists Ltd.

LADHA, S., KINGSTON, C.A., BOWLER, K & MANNING, R. (1989) Effect of fatty acid supplementation on the thermal sensitivity of HTC cells in culture. Biochem Soc T. 17 530-531

LEITH, J. T., MILLER, R. C., GERNER, E. W. & BOONE, M. L. M. (1977) Hyperthermic potentiation: biological aspects and applications to radiation therapy, *Cancer Res.* 39 766–779.

LEPOCK, J. R., MASSICOTTE-NOLAN, P., RULE, G. S. & KRUUV, J. (1981) Lack of correlation between hyperthermic cell killing, thermotolerance and membrane lipid fluidity, *Radiat. Res.* 87 300–313.

LEPOCK, J. R. (1982) Involvement of membranes in cellular responses to hyperthermia, *Radiat. Res.* 92 433–438.

LEPOCK, J. R., CHENG, K.-H., AL-QYSI, H. & KRUUV, J. (1983) Thermotropic lipid and protein transitions in Chinese hamster lung cell membranes: relationship to hyperthermic cell killing, *Can. J. Biochem.* 61 421–427.

LEPOCK, J. R., FREY, H. E., RODAHL, A. M. & KRUUV, J. (1988) Thermal analysis of CHL V79 cells using differential scanning calorimetry: implications for hyperthermic cell killing and the heat shock response, *J. Cell Physiol.* 137 14–24.

LEPOCK, J. R., FREY, H. E., BAYNE, H. & MARKUS, J. (1989) Relationship of hyperthermia-induced hemolysis of human erythrocytes to the thermal denaturation of membrane proteins, *Biochim. Biophys. Acta* 980 191–201.

LEVINE, E. M. & ROBBINS, E. B. (1970) Differential temperature sensitivity of normal and cancer cells in culture, *J. Cell Physiol.* 76 373–379.

LEYKO, W. & BARTOSZ, G. (1986) Review: membrane effects of ionizing radiation and hyperthermia, *Int. J. Radiat. Biol.* 49 743–770.

LI, G. C. & HAHN, G. M. (1978) Ethanol-induced tolerance to heat and adriamycin, *Nature* 274 699–701.

LI, G. C. & HAHN, G. M. (1980) Adaptation to different growth temperatures modifies some mammalian cell survival responses, *Exp. Cell Res.* 128 475–485.

LI, G. C., SHIU, E. C. & HAHN, G. M. (1980) Similarities in cellular inactivation by hyperthermia or by ethanol, *Radiat. Res.* 82 257–268.

LIN, P. S., WALLACH, D. F. H. & TSAI, S. (1973) Temperature induced variations in the surface topology of cultured lymphocytes as revealed by scanning electron microscopy, *Proc. Natn. Acad. Sci. (USA)* 70 2492–2496.

LIN, P. S., BUTTERFIELD, C. E. & WALLACH, D. F. H. (1977) Hyperthermic treatment ($43°C$) rapidly impedes attachment of fibroblasts to culture substrates, *Cell Biol. Int. Rep.* 1 57–61.

LIN, P. S., KWOCK, L., HEFTER, K. & WALLACH, D. F. H. (1978) Modification of rat thymocyte membrane properties by hyperthermia and ionising radiation, *Int. J. Radiat. Biol.* 33 371–382.

LOPEZ-SAURA, P., TROUET, A. & TULKENS, P. (1978) Analytical fractionation of cultured hepatoma cells (HTC cells), *Biochim. Biophys. Acta* 543 430–449.

LOTEN, E. G. & REDSHAW-LOTEN, J. C. (1986) Preparation of rat liver plasma membranes in a high yield, *Analyt. Biochem.* 154 183–185.

LOW, P. S., LLOYD, D. H., STEIN, T. S. & ROGERS, J. A. (1979) Calcium displacement by local anaesthetics: dependence on *pH* and anaesthetic charge, *J. Biol. Chem.* 254 4119–4125.

MACHTIGER, N. A. & FOX, C. F. (1973) Biochemistry of bacterial membranes, *Ann. Rev. Biochem.* 42 575–600.

MAGUN, B. E. & FENNIE, C. E. (1981) Effects of hyperthermia on binding, internalisation and degradation of epidermal growth factor, *Radiat. Res.* 86 133–146.

MAHER, P. & SINGER, S. J. (1984) Structural changes in membranes produced by the binding of small amphipathic molecules, *Biochem.* 23 232–240.

MAHLER, S. M., WILCE, P. A. & SHANLEY, B. C. (1988) Studies on regenerating liver and hepatoma plasma membranes. I. Lipid and protein composition, *Int. J. Biochem.* 20 605–611.

MAKMAN, M. H. (1971) Conditions leading to enhanced response to glucogon, epinephrine or prostaglandins by adenylate cyclase of normal and malignant cultured cells, *Proc. Natn. Acad. Sci. (USA)* 68 2127–2130.

MANNING, R., CHAMBERS, J., LAHDA, S., KINGSTON, C. A. & BOWLER, K. (1989) Rapid purification of plasma membranes in high yield from HTC cells, *Bioch. Soc. T.* 17 670–671.

MARRA, C. A., ALANIZ, M. J. T. de. & BRENNER, R. R. (1984) Uptake and metabolism of eicosa-8,11,14-trienoic acid in normal hepatocytes and HTC cells, *Mol. Cell. Biochem.* 65 143–152.

MARTIN, C. E. & THOMPSON, G. A. Jnr. (1978) Use of fluorescence polarisation to monitor intracellular membrane changes during temperature acclimation. Correlation with lipid compositional and ultrastructural changes, *Biochem.* 17 3581–3586.

MASSICOTTE-NOLAN, P., GLOFCHESKI, D. J., KRUUV, J. & LEPOCK, J. R. (1981) Relationship between hyperthermic cell killing and protein denaturation by alcohols, *Radiat. Res.* 87 284–299.

McATEER, J. A. & DOUGLAS, W. H. J. (1979) Monolayer culture techniques, *Cell culture, section 1: Basic methods in enzymology*, eds. Jakoby, W. B. & Pastan, I. H. Vol. LVIII pp132–140.

McKEEL, D. W. & JARETT, L. (1970) Preparation and characterisation of a plasma fraction from isolated fat cells, *J. Cell Biol.* 44 417–432.

MERED, B., ALBRECHT, P. & HOPPS, H. E. (1980) Cell growth optimisation in microcarrier culture, *In Vitro* 16 859–865.

MITCHELL, K. J. & WRAY, W. (1979) Mitotic cell populations obtained from a microcarrier culturing system, *Exp. Cell Res.* 123 452–455.

MONDOVI, B., STROM, R., ROTILIO, G., FINAZZI, A. A., CAVALIERE, R. & ROSSI FANELLI, A. (1969) The biochemical mechanism of selective heat sensitivity of cancer cells. I. Studies on cellular respiration, *Eur. J. Cancer* 5 129–136.

MONNERON, A., BLOBEL, G. & PALADE, G. E. (1972) Fractionation of the nucleus by divalent cations. Isolation of nuclear membranes, *J. Cell Biol.* 55 104–125.

MORRIS, H. P. (1965) *Adv. Cancer Res.* 9 227–302.

MOSCOWITZ, M. S. (1967) *Lipid metabolism in tissue culture cells*, eds. Rothblat, G. H. & Kritchevsky, Y. D., Wistar Institute Press, Philadelphia, pp49–62.

MOSMANN, T. (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays, *J. Immunol. Methods* 65 55–63.

MUCKLE, D. S. & DICKSON, J. A. (1971) The selective inhibition effect of hyperthermia on the metabolism and growth of malignant cells, *Br. J. Cancer* 25 771.

MULCAHY, R. T., GOULD, M. N., HIDVERGI, E., ELSON, C. E. & YATVIN, M. B. (1981) Hyperthermia and surface morhology of P388 ascites tumour cells: effects on membrane modifications, *Int. J. Radiat. Biol.* 39 95–106.

NEEDLEMAN, S. W., SPECTOR, A. A. & HOAK, J. C. (1982) Enrichment of human platelet phospholipids with linoleic acid diminishes thromboxane release, *Prostaglandis* 24 607–621.

NELSON, G. J., KELLY, D. S. & HUNT, J. E. (1986) Effect of nutritional status on the fatty acid composition of rat liver and cultured hepatocytes, *Lipids* 21 454–459.

NEUDOERFFER, T. S. & LEA, C. H. (1967) Effects of dietary polyunsaturated fatty acids on the composition of the individual lipids of turkey breast and leg muscle, *Br. J. Nutr.* 21 691–714.

NEVILLE, D. M. (1960) The isolation of a cell membrane fraction from rat liver, *J. Biophys. Biochem. Cytol.* 8 413–422.

NICOLSEN, G. L., SMITH, J. R. & POSTE, G. (1976) Effects of local anaes-

thetics on cell morphology and membrane associated cytoskeletal organisation in BALB/3T3 cells, *J. Cell Biol.* 68 395–402.

NIELSEN, O. S. & OVERGAARD, J. (1979) Effect of extra-cellular *pH* on thermotolerance and recovery of hyperthermic damage in vitro *Cancer Res.* 39 2772–2778.

NIELSEN, O. S. & OVERGAARD, J. (1982) Influence of time and temperature on the kinetics of thermotolerance in L_1A_2 cells in vitro, *Cancer Res.* 42 4190–4196.

NINNO, R. E., De TORRENGO, M. A. P., CASTUMA, J. C. & BRENNER, R. R. (1974) Specificity of 5- and 6-fatty acid desaturases in rat and fish, *Biochim. Biophys. Acta* 360 124-133.

NYSTROM, R. A. (1973) in *Membrane Physiology*, New Jersey, Prentice-Hall, pp6–62.

OHKI, S. (1984) Adsorption of local anaesthetics on phospholipid membranes, *Biochim. Biophys. Acta* 777 56–66.

OLDFIELD, E. & CHAPMAN, D. (1972) Dynamics of lipids in membranes: heterogeneity and the role of cholesterol, *FEBS Lett.* 23 285–297.

OLMSTED, C. A. (1967) A physico-chemical study of fetal calf sera used as tissue culture nutrient correlated with biological tests for toxicity, *Exp. Cell Res.* 48 283–299.

OP den KAMP, J. A. F. (1979) Lipid asymmetry in membranes, *Ann. Rev. Biochem.* 48 47–71.

OTTE, J. (1988) Hyperthermia in cancer therapy, *Eur. J. Pediatr.* 147 560–569.

OVERATH, P., SCHAIRER, H. U. & STOFFEL, W. (1970) Correlation of in vivo and in vitro phase transitions of membrane lipids in *Escherichia coli*, *Proc. Natn. Acad. Sci. (USA)* 67 606–612.

OVERGAARD, J. & OVERGAARD, K. (1975) Effect of environmental acidity on the hyperthermic treatment of tumour cells, *Int. Res. Commun. Syst. Med. Sci.: Biochem. Cancer Membr. Biol.* 3 386.

OVERGAARD, J. & POULSON, H. S. (1977) Effect of hyperthermia and environmental acidity on the proteolytic activity in murine ascites tumour cells, *J. Natl. Cancer Inst.* 58 1159–1161.

PALZER, R. J. & HIEDELBERGER, C. (1973) Influence of drugs and synchrony on the hyperthermic killing of HeLa cells, *Cancer Res.* 33 422–427.

PAPAHADJOPOULOS, D., JACOBSON, K., POSTE, G. & SHEPHERD, G. (1975) Effects of local anaesthetics on membrane properties. I. Changes in the

fluidity of phospholipid bilayers, *Biochim. Biophys. Acta* 394 504–519.

PARKS, L. C. & SMITH, G. V. (1983) Systemic hyperthermia by extracorporeal induction: techniques and results, in *Hyperthermia and Cancer Treatment*, ed. Storm, F. K., Hall Medical Publishers, Boston, pp407–446.

PAYRASTRE, B., PLANTAVID, M., ETIEVAN, C., RIBBES, G., CARRATERO, C., CHAP, H. & DOUSTE-BLAZY, L. (1988) Characterisation of plasma membranes from A431 cells, isolated by self generating Percoll gradient: a rapid isolation procedure to obtain plasma membranes with functional epidermal growth factor receptors, *Biochim. Biophys. Acta* 939 355–365.

PEARLMAN, D. (1979) Use of antibiotics in cell culture media, *Cell culture, section 1: Basic methods in enzymology*, eds. Jakoby, W. B. & Pastan, I. H. Vol. LVIII pp110–116.

PETTIGREW, R. T., GALT, J. M., LUDGATE, C. M., HORN, D. B. & SMITH, A. N. (1974) Circulatory and biochemical effects of whole-body hyperthermia, *Br. J. Surg.* 61 727–730.

PHAIRE-WASHINGTON, L., HOWARD-ROBINSON, G., LUCAS, J., ROBERSON, J., BRAKO, E., McNEIL, N., WILLIAMS, S., WATSON, C., IGBO, T., BRAKO, L., FARSHORI, P. & CANNON, V. (1987) The intermediate filament skeleton of macrophages, *Scanning Microsc.* 1 775–782.

PHARMACIA FINE CHEMICALS A. B. (1981) Microcarrier cell culture: principles and methods, *Technical booklet series*, Uppsala, Sweden.

POON, R., RICHARDS, J. M. & CLARK, W. R. (1981) The relationship between plasma membrane lipid composition and physical-chemical properties. II. Effect of phospholipid fatty acid modulation on plasma membrane physical properties and enzymic activities, *Biochim. Biophys. Acta* 649 58–66.

PORRO, G., TAGLIABUE, E., MENARD, S. & COLNAGHI, M. I. (1986) Influence of in vitro culture conditions on tumour cell differentiation, *Tumori* 72 15–20.

POSTE, G. & REEVE, P. (1972) Inhibition of cell fusion by local anaesthetics and tranquillizers, *Exp. Cell Res.* 72 556–560.

POZNANSKY, M., KIRKWOOD, D. & SOLOMAN, A. K. (1973) Modulation of red cells (K^+) transport by membrane lipids, *Biochim. Biophys. Acta* 330 351–355.

PUCK, T. T., MARCUS, P. I. & CIECIURA, S. J. (1955) Clonal growth of mammalian cells in vitro. Growth characteristics of colonies from single HeLa cells with and without a 'feeder' layer, *J. Exp. Med.* 103 273–284.

PUCK, T. T. & MARCUS, P. I. (1956) Action of X-rays on mammalian cells, *J. Exp. Med.* 103 653–666.

RAAPHORST, G. P., ROMANO, S. L., MITCHELL, J. B., BEDFORD, J. S. & DEWEY, W. C. (1979) Intrinsic differences in heat and/or X-ray sensitivity of seven mammalian cell lines cultured and treated under identical conditions, *Cancer Res.* 39 369–401.

RAAPHORST, G. P., VADASZ, J. A., AZZAM, E. I., SARGENT, M. D., BORSA, J. & EINSPENNER, M. (1985) Comparison of heat and/or radiation sensitivity and membrane composition of seven X-ray-transformed *C3H* $10T\frac{1}{2}$ cell lines and normal *C3H* $10\frac{1}{2}$ cells, *Cancer Res.* 45 5452–5456.

RAHEJA, R. K., KAUR, C., SINGH, A. & BHATIA, I. S. (1973) New colorimetric method for the quantitative estimation of phospholipids without acid digestion, *J. Lipid Res.* 14 695–697.

RECORD, M., BES, J. C., CHAP, H. & DOUSTE-BLAZY, L. (1982) *Biochim. Biophys. Acta* 688 57–65.

RICHTER, A. (1970) Improved medium for the cultivation of differential liver cells, *In Vitro* 6 220–221.

RICHTER, A., SANFORD, K. K. & EVANS, V. J. (1972) Influence of oxygen and culture media on plating efficiency of some mammalian tissue cells, *J. Natl. Cancer Inst.* 49 1705–1712.

ROFSTAD, E. K. & BRUSTAD, T. (1984) Response to heat treatment ($42.5°C$) in vivo and in vitro of five human melanoma xenografts, *Br. J. Radiol.* 57 1023–1032.

ROME, L. H., GARVIN, A. J., ALLIETA, M. M. & NEUFELD, E. F. (1979) Two species of cysosonal organelles in cultured human fibroblasts, *Cell* 17 143–153.

ROSENTHAL, M. D. (1980) Selectivity in incorporation, utilisation and retention of oleic and linoleic acids by human skin fibroblasts, *Lipids* 15 838–848.

ROSENTHAL, M. D. (1987) Fatty acid metabolism of isolated mammalian cells, *Prog. Lipid Res.* 26 87–124.

ROTH, S. & SEEMAN, P. (1971) All lipid-soluble anaesthetics protect red cells, *Nat. New Biol.* 231 284–285.

ROTHBLAT, G. H., ARGOGAST, L. Y., OUELLETTE, L. & HOWARD, B. V. (1976) Preparation of delipidised serum protein for use in cell culture systems, *In Vitro* 12 554.

ROTI ROTI, J. L. & WINWARD, R. T. (1980) Factors affecting the heat induced increase in protein content of chromatin, *Radiat. Res.* 81 138–144.

ROTI ROTI, J. L. (1982) Heat induced cell death and radiosensitisation: Molecular mechanics, *Natl. Cancer Inst. Monogr.* 61 3–10.

ROTI ROTI, J. L. & WILSON, C. F. (1984) The effects of alcohols, procaine

and hyperthermia on the protein content of nuclei and chromatin, *Int. J. Radiat. Biol.* 46 25–33.

RUDLAND, P. S., TWISTON-DAVIES, A. & WARBURTON, M. J. (1982) Prostaglandin-induced differentiation or DMSO-induced differentiation: reduction of the neoplastic potential rat mammary tumour stem-cell line, *J. Natl. Cancer Inst.* 69 1083–1093.

RUIFROK, A. C. C., KANON, B., & KONINGS, A. W. T. (1985) Correlation of colony forming ability of mammalian cells with potassium content after hyperthermia under different experimental conditions, *Radiat. Res.* 103 452–454.

SABINE, J. R. (1983) Membrane homeostasis: is there an optimal level of membrane cholesterol? *Biosci. Rep.* 3 337–344.

SAMULSKI, T. V., LEE, E. R. & HAHN, G. M. (1984) Hyperthermia as a clinical treatment modality, *Cancer Treat. Rep.* 68 309–316.

SAPARETO, S. A., HOPWOOD, L. E., DEWEY, W. C., RAJU, M. R. & GRAY, J. W. (1978) Effects of hyperthermia on survival and progression of Chinese hamster ovary cells, *Cancer Res.* 38 393–400.

SAUVAGE, P., LOPEZ-SAURA, P., LEROY-HOUYET, M. A., TULKENS, P. & TROUET, A. (1981) Analytical characterisation and purification of plasma membrane from cultured hepatoma cells (HTC cells), *Biochim. Biophys. Acta* 644 41–52.

SCHAMHART, D. H. J., BERENDSEN, W., Van RIJN, J. & Van WIJK, R. (1984) Comparative studies of heat sensitivty of several rat hepatoma cell lines and hepatocytes in primary culture, *Cancer Res.* 44 4507–4516.

SCHAMHART, D. H. J., Van WALRAVEN, H. S., WIEGANT, F. A. C., LINNEMANS, W. A. M., Van RIJN, J., Van Den BERG, J. & Van WIJK R. (1984) Thermotolerance in cultured hepatoma cells: cell viability, cell morphology, protein synthesis and heat-shock proteins, *Radiat. Res.* 98 82–95.

SCHERER, W. F. & HOOGASIAN, A. C. (1954) Preservation at subzero temperatures of mouse fibroblasts (strain L) and human epithelial cells (strain HeLa), *Proc. Soc. Exp. Biol. Med.* 87 480–487.

SCHLIWA, M. (1986) *The Cytoskeleton*, eds. Alfert, M., Goldstein, L. & Beerman, W., Springer-Verlag, Vienna-New York, pp1–323.

SCHMIDT-NIELSON, K. (1979) *Animal Physiology Adaptation and Environment*, second edition, Cambridge University press.

SCHMITT, K., DAEUBENER, W., BITTER-SUERMANN, D. & HADDING, U. (1988) A safe and efficient method for elimination of cell culture mycoplasmas using ciprofloxacin, *J. Immunol. Methods* 109 17–26.

SCHREK, R., CHANDRA, S., MOLNAR, Z. & STEFANI, S. S. (1980) Two types of interphase death of lymphocytes exposed to temperatures of 37-45°C, *Radiat. Res.* 82 162–170.

SCHROEDER, F. (1983) Lipid domains in plasma membranes from rat liver, *Eur. J. Biochem.* 132 509–516.

SCHULMAN, N. & HALL, E. J. (1974) Hyperthermia: its effect on proliferative and plateau phase cell cultures, *Radiol.* 113 209–211.

SCHNEEBERGER, E. E., LYNCH, R. D. & GEYER, R. P. (1971) Formation and disappearance of triglyeride droplets in strain L fibroblasts: an electron microscopic study, *Exp. Cell Res.* 69 193–206.

SEEMAN, P. (1972) The membrane actions of anaesthetics and tranquilisers, *Pharmacol. Rev.* 24 583–633.

SELLINGER, O. Z., BEAUFAY, H., JAQUES, P., DOYEN, A. & De DUVE, C. (1960) Intracellular distribution and properties of β-N-acetylglucosaminidase and β-galactosidase in rat liver, *Biochem. J.* 74 450–456.

SERTICH, G. J., FULLER, D. J. M. & GERNER, E. W. (1980) Biochemical studies on the thermal sensitivity of Chinese hamster cells growing in defined medium, *Natl. Cancer Inst. Monogr.* 61 81–83.

SHEETZ, M. P. & SINGER, S. J. (1974) Biological membranes as bilayer couples. A molecular mechanism of drug-erythrocyte interactions, *Proc. Natn. Acad. Sci. (USA)* 71 4457–4461.

SHINITZKY, M. (1984) in *Physiology of membrane fluidity*, Vol. 1, CRC Press, Florida, pp99–129.

SILBERT, D. F., CRONAN, J. E. Jnr., BEACHAM, I. R. & HARDER, M. E. (1974) Genetic engineering of membrane lipid, *Fed. P.* 33 1725–1732.

SILVESTRINI, B., HAHN, G. M., CIOLI, V. & DeMARTIN, C. (1983) Effects of ionidamine alone or combined with hyperthermia in some experimental cell and tumour systems, *Br. J. Cancer* 47 221–231.

SIMON, E. W. (1974) Phospholipids and plant membrane permeability, *New. Pythol.* 73 377–420.

SIMPSON, I. A. & SONNE, O. (1982) A simple, rapid and sensitive method for measuring protein concentration in subcellular membrane fractions prepared by sucrose density ultracentrifugation, *Analyt. Biochem.* 119 424–427.

SINENSKY, M. (1974) Homeoviscous adaptation - A homeostatic process that regulates the viscosity of memebrane lipids in *Escherichia Coli*, *Proc. Natn. Acad. Sci. (USA)* 71 522–525.

SINGER, S. J. & NICHOLSON, G. L. (1972) The fluid mosaic model of the

structure of cell membranes, *Science* 175 720–731.

SINGER, M. A. & JAIN, M. K. (1980) Interaction of four local anaesthetics with phospholipid bilayer membranes: permeability effects and possible mechanisms, *Can. J. Biochem.* 58 815–821.

SIRICA, A. E., RICHARDS, W., TSUKADA, Y, SATTLER, C. A. & PITOT, H. C. (1979) Fetal phenotYpic expression by adult rat hepatocytes on collagen gel/nylon meshes, *Proc. Natn. Acad. Sci. (USA)* 76 283–287.

SLATER, T. F., SAWYER, B., & STRAULI, U. (1963) Studies on succinate-tetrazolium reductase systems. III. Points of coupling of four different tetrazolium salts, *Biochim. Biophys. Acta* 77 383–393.

SLUSSER, H., HOPWOOD, L. E. & KAPISZEWSKA, M. (1982) Inhibition of membrane transport by hyperthermia, *Natl. Inst. Cancer Monogr.* 61 85–87.

SMITH, I. C. P. & BUTLER, K. W. (1985) *Effects of Anesthesia*, eds. Corino, B. G. et al., Waverley, Baltimore, pp1–11.

SPECTOR, A. A. & HOAK, J. C. (1969) An improved method for the addition of long chain free fatty acid to protein solutions, *Analyt. Biochem.* 32 297–302.

SPECTOR, A. A., KISER, R. E., DENNING, G. M., KOH, S.-W. & DeBAULT, L. E. (1979) Modification of the fatty acid composition of cultured human fibroblasts, *J. Lipid Res.* 20 536–547.

SPECTOR, A. A., HOAK, J. C., FRY, G. L., DENNING, G. M., STOLL, L. L. & SMITH, J. B. (1980) Effect of fatty acid modification on prostacyclin production by cultured human endothelial cells, *J. Clin. Inv.* 65 1003–1012.

SPECTOR, A. A. & YOREK, M. A. (1985) Membrane lipid composition and cellular function, *J. Lipid Res.* 26 1015–1035.

STEEL, G. G. (1977) *Growth kinetics of tumours*, Clarendon Press, Oxford.

STEVENSON, M. A., MINTON, K. W. & HAHN, G. M. (1981) Survival and concanavalin-A-induced capping in CHO fibroblasts after exposure to hyperthermia, ethanol and X-irradiation, *Radiat. Res.* 86 467–478.

STEVENSON, A., GALEY, W. R. & TOBEY, R. A. (1983) Hyperthermia-induced increase in potassium transport in Chinese hamster cells, *J. Cell Physiol.* 115 75–86.

STEVENSON, M. A., CALDERWOOD, S. K. & HAHN, G. M. (1986) Rapid increases in inositol triphosphate and intracellular calcium after heat shock, *Biochem. Biophys. Res. Commun.* 137 826–833.

STEWART, J. R. (1988) Prospects for hyperthermia in cancer therapy, *Hyperthermia Oncol.* 1 1–12.

STORCH, J. & SCHACHTER, D. (1984) Dietary induction of acyl chain desaturates alters the lipid composition and fluidity of rat hepatocyte plasma membranes, *Biochem.* 23 1165–1170.

STROM, R., CRIFO, C., ROSSI-FANELLI, A. & MONDOVI, B. (1977) Biochemical apects of heat sensitivity of tumour cells, in *Selective Heat Sensitivity of Cancer Cells*, eds. Rossi-Fanelli, A., Cavaliere, R., Mondovi, B. & Morricca, G., Springer Verlag, Berlin Heidelberg New York, pp7–35.

STUBBS, C. D., TSANG, W. M., BELIN, J., SMITH, A. D. & JOHNSON, S. M. (1980) Incubation of exogeneous fatty acids with lymphocytes. Changes in fatty acid composition and effects on the rotational relaxation time of 1,6-diphenyl-1,3,5-hexatriene, *Biochem.* 19 2756–2762.

STUBBS, C. D. (1983) Membrane fluidity: structure and dynamics of membrane lipids, in *Essays in Biochemistry*, eds. Campbell, P. N. & Marshall, R. D., London Academic Press, Vol. 19 pp1–39.

STUBBS, C. D. & SMITH, A. D. (1984) The modification of mammalian membrane polyunsaturated fatty acid composition in relation to membrane fluidity and function, *Biochim. Biophys. Acta* 779 89–137.

SUNDLER, R. & ÅKESSON, B. (1975) Regulation of phospholipid biosynthesis in isolated rat hepatocytes. Effects of different substrates, *J. Biol. Chem.* 250 3359.

SUREWICZ, W. K. & LEYKO, W. (1982) Interaction of local anaesthetics with model phospholipid membranes. The effect of *pH* and phospholipid composition studied by quenching of an intramembrane fluorescent probe, *J. Pharm. Pharmacol.* 34 359–363.

SWEET, W. D. & SCHROEDER, F. (1986) Charged anaesthetics alter LM-fibroblast plasma membrane enzymes by selective fluidisation of inner or outer membrane leaflets, *Biochem. J.* 239 301–310.

SWEET, W. D. & SCHROEDER, F. (1988) Polyunsaturated fatty acids alter sterol transbilayer domains in lm fibroblast plasma membrane, *FEBS Lett.* 229 188–192.

SWIM, H. E., HALL, R. F. & PARKER, R. F. (1958) Some practical aspects of storing mammalian cells in the dry-ice chest, *Cancer Res.* 18 711–717.

SYMONDS, R. P., WHELDON, T. E., CLARK, B. & BAILEY, G. (1981) A comparison of the response to hyperthermia of murine haemopoietic stem cells (CFU-5) and L1210 leukemia cells: enhanced killing of leukemic cells in the presence of normal marrow cells, *Br. J. Cancer* 44 682–691.

TER HAAR, G. (1986) Effects of increased temperature on cells, on membranes and on tissues, in *Hyperthermia*, eds. Watmough, D. J. & Ross, W. M., Blackie

& son Ltd., pp14–41.

THILLY, N. G. & LEVINE, D. W. (1979) Microcarrier culture: a homogeneous environment for studies of cellular biochemistry, *Cell culture, section 1: Basic methods in enzymology*, eds. Jakoby, W. B. & Pastan, I. H. Vol. LVIII pp184–194.

THOMPSON, E. B., TOMKINS, G. M. & CURRAN, J. F. (1966) Induction of tyrosine and α-ketoglutarate transamininase by steroid hormones in a newly established tissue culture cell line, *Proc. Natn. Acad. Sci. (USA)* **56** 296–303.

THOMPSON, B. E., ANDERSON, C. U. & LIPPMAN, M. E. (1975) Serum-free growth of HTC cells containing glucocorticoid- and insulin-inducible tyrosine aminotransferase and cytoplasmic glucocorticoid recepters, *J. Cell Physiol.* **86** 403–412.

TOMASOVIC, S. P., TURNER, G. N. & DEWEY, W. C. (1978) Effect of hyperthermia on nonhistone proteins isolated with DNA, *Radiat. Res.* **73** 535–552.

TOVEY, K. C., OLDHAM, K. G. & WHELAN, J. A. M. (1974) A simple direct assay for cyclic AMP in plasma and other biological samples using an improved competitive protein binding technique, *Clin. Chim. Acta* **56** 221–234.

TRUANT, P. & TAKMAN, B. (1965) *Drills Pharmacology in Medicine*, 3rd edition, ed. Dipalma, J. F., McGraw-Hill, New-York, p148.

TSAI, C.-M., CHEN, K. U. & CANELLAKIS, E. S. (1975) Isolation and characterisation of the plasma membrane of L-1210 cells. Iodination as a marker for the plasma membrane, *Biochim. Biophys. Acta* **401** 196–212.

TWETO, J., FRIEDMAN, E. & DOYLE, D. (1976) Proteins of the hepatoma tissue culture cell plasma membrane, *J. Supramol. Struct.* **4** 141–159.

UPRETI, G. C., DEANTUENO, R. J. & WOOD, R. (1983) Membrane lipids of hepatic tissue. 1. Neutral lipids from subcellular fractions of liver and hepatoma, *J. Natl. Cancer Inst.* **70** 559–566.

URADE, R. & KITO, M. (1982) Perturbation of lipid metabolism by palmitic acid in Chinese hamster V79-R cells, *J. Biochem.* **91** 1639–1649.

Van BLITTERSWIJK, W. J., Van Der MEER, B. W. & HILKMAN, H. (1987) Quantitative contributions of cholesterol and the individual classes of phospholipids and their degree of fatty acyl (un)saturation to membrane fluidity measured by fluorescence polarisation, *Biochem.* **26** 1746–1756.

Van DEENEN, L. M. M. (1971) Chemistry of phospholipids in relation to biological membranes, *Pure App. Chem.* **25** 25–56.

Van DONGEN, G. & Van WIJK, R. (1986) Effect of serum on heat response of synchronised mouse neuroblastoma cells: protection of cell cycle progression,

protein synthesis and survival, *Int. J. Radiat. Biol.* 50 77–91.

Van WEZEL, A. L. (1967) Growth of cell strains and primary cells on microcarriers in homogenous culture, *Nature* 216 64–65.

VARANI, J., BENDELOW, M. J., CHUN, J. H. & HILLEGAS, W. A. (1986) Cell growth on microcarriers: comparison of proliferation on and recovery from various substrates, *J. Biol. Stand.* 14 331–336.

VAUPEL, P. (1979) Oxygen supply to malignant tumours, in *Tumour Blood Circulation: Angiogenesis, Vascular Morphology and Blood Flow of Experimental and Human Tumours*, ed. Peterson, H. I., Boca Raton, Florida, CRC press, pp143–168.

VIDAIR, C. A. & DEWEY, W. C. (1986) Evaluation of a role for intracellular Na^+, K^+, Ca^{2+} and Mg^{2+} in hyperthermic killing, *Radiat. Res.* 105 187–200.

VIDAIR, C. A. & DEWEY, W. C. (1988) Two distinct modes of hyperthermic cell death, *Radiat. Res.* 116 157–171.

Von HOFF, D. D., FORSETH, B. J., HUONG, M., BUCHOK, J. B. & LATHAN, B. (1986) Improved plating efficiencies for human tumours closed in capillary tubes versus petri dishes, *Cancer Res.* 46 4012–4017.

WALLACH, D. F. H. & ULLREY, D. (1962) The hydrolysis of ATP and related nucleotides by ehrlich ascites carcinoma cells, *Cancer Res.* 22 228–234.

WALLACH, D. F. H. & KAMAT, V. B. (1966) Preparation of plasma-membrane fragments from mouse ascites tumour cells, *Methods Enzymol.* 8 164–172.

WARREN, S. L. (1935) Preliminary study of the effect of artificial fever upon hopeless tumour cases, *Am. J. Roentgenol.* 33 75–87.

WARTERS, R. L. & HENLE, K. J. (1982) DNA degradation in Chinese hamster ovary cells after exposure to hyperthermia, *Cancer Res.* 42 4427–4432.

WEBER, K. & GROSCHEL-STEWART, U. (1974) Antibody to myosin: the specific visualisation of myosin-containing filaments in nonmuscle cells, *Proc. Natn. Acad. Sci. (USA)* 71 4561–4564.

WELCH, W. J. & SUHAN, J. P. (1985) Morphological study of the mammalian stress response: characterisation of changes in the cytoplasmic organelles, cytoskeleton, and nucleoli, and appearance of intranuclear Actin filaments in rat fibroblasts after heat-shock treatment, *J. Cell Biol.* 101 1198–1211.

WESTERMARK, N. (1927) The effect of heat upon rat tumours, *Skand. Arch. Physiol.* 52 257–322.

WESTRA, A. & DEWEY, W. C. (1971) Variation in sensitivity to heat shock during the cell cycle of Chinese hamster cells in vitro, *Int. J. Radiat. Biol.* 19 467–477.

WESTWOOD, J. T., CHURCH, R. B. & WAGENAAR, E. B. (1985) Changes in protein phosphorylation during the cell cycle of Chinese hamster ovary cells, *J. Biol. Chem.* 260 10308–10313.

WHEATLEY, D. N., KERR, C. & GREGORY, D. W. (1989) Heat induced damage to HeLa S_3 cells: correlation of viability, permeability, osmosensitivity, phase-contrast light-, scanning electron- and transmission electron-microscopical findings, *Int. J. Hyperthermia* 5 145–162.

WHITE, D. A. (1973) in *Form and function of phospholipids*, eds. Ansell, G. B., Dawson, R. M. C. & Hawthorne, J. N., Elsevier, Amsterdam, pp441–482.

WHITFIELD, J. F., MACMANUS, J. P., RIXON, R. H., BOYNTON, A. L., YOUDALE, T. & SWIERENGA, S. (1976) The positive control of cell proliferation by the interplay of calcium ions and cyclic nucleotides: a review, *In Vitro* 12 1–18.

WIEGAND, R. D. & WOOD, R. (1974) Lipids of cultured hepatoma cells: VI. Glycerolipid and monoenoic fatty acid biosynthesis in minimal derivation hepatoma 7288C, *Lipids* 10 194–201.

WIEGANT, F. A. C., TUYL, M. & LINNEMANNS, W. A. M. (1985) Calmodulin inhibitors potentiate hyperthermic cell killing, *Int. J. Hyperthermia* 1 157–169.

WIKE-HOOLEY, J. L., HAVEMAN, J. & REINHOLD, H. S. (1984) The relevance of tumour *pH* to the treatment of malignant disease, *Radiother. Oncol.* 2 343–366.

WILLIAMS, R. E., WISNIESKI, B. J., RITTENHOUSE, H. G. & FOX, C. F. (1974) Utilisation of fatty acid supplements by cultured cells, *Biochem.* 13 1969–1977.

WOLTERS, H. & KONINGS, A. W. T. (1984) Radiosensitivity of normal and polyunsaturated fatty acid supplemented fibroblasts after depletion of glutathione, *Int. J. Radiat. Biol.* 46 161–168.

WONG, R. S. L. & DEWEY, W. C. (1982) Molecular studies on the hyperthermia inhibition of DNA synthesis in Chinese hamster ovary cells, *Radiat. Res.* 92 370–395.

WONG, R. S. L. & DEWEY, W. C. (1983) Molecular mechanisms for enhancement of hyperthermic damage by procaine, *HCl* and low *pH* treatments, *Tumour Biology and Therapy*, eds. Broerse, J. J., Barendsen, G. W., Kal, H. B. & Van der Kogel, A. J., Amsterdam: Martinus Nijhoff, D6–60.

WOOD, R. (1973) Lipids of cultured hepatoma cells: 1. Effect of serum lipid levels on cell and media lipids, *Lipids* 8 690–701.

WOOD, R. & FALCH, J. (1973) Lipids of cultured hepatoma cells: II. Effect of

media lipids on cellular phospholipids, *Lipids* 8 702–710.

WOODCOCK, C. L. F. (1980) Nucleus-associated intermediate filaments from chicken erythrocytes, *J. Cell Biol.* 85 881–889.

YATVIN, M. B. (1977) The influence of membrane lipid composition and procaine in hyperthermic death of cells, *Int. J. Radiat. Biol.* 32 513–521.

YATVIN, M. B., CLIFTON, K. H. & DENNIS, W. H. (1979) Hyperthermia and local anaesthetics: potentiation of survival of tumour-bearing mice, *Science* 205 195–196.

YATVIN, M. B., CREE, T. C., ELSON, C. E., GIPP, J. J., TEGMO, P. M. & VORPAHL, J. W. (1982) Probing the relationship of membrane 'fluidity' to heat killing of cells, *Radiat. Res.* 89 644–646.

YATVIN, M. B., GIPP, J. J., RUSY, B. F. & DENNIS, W. H. (1982) Correlation of bacterial hyperthermic survival with anaesthetic potency, *Int. J. Radiat. Biol.* 42 141–149.

YATVIN, M. B., ABUIRMEILEH, N. M., VORPAHL, J. W. & ELSON, C. E. (1983) Biological optimisation of hyperthermia: modification of tumour membrane lipids, *Eur. J. Cancer Clin. Oncol.* 19 657–663.

YATVIN, M. B., DENNIS, W. H., ELEGBEDE, J. A. & ELSON, C. E. (1987) Sensitivity of tumour cells to heat and ways of modifying the response, in *Temperature and Animal Cells*, S.E.B. symposium XLI, eds. Bowler, K. & Fuller, B. J., Cambridge: The Company of Biologists Ltd.

YAU, T. M. (1979) Procaine-mediated modification of membranes and of the response to X-irradiation and hyperthermia in mammalian cells, *Radiat. Res.* 80 523–541.

YI, P. N., CHANG, C. S., TALLEN, M., BAYER, W. & BALL, S. (1983) Hyperthermia induced intracellular ionic level changes in tumour cells, *Radiat. Res.* 93 534–544.

YOUNGNER J. S. (1954) Monolayer tissue cultures I. Preparation and standardization of suspensions of trypsin-dispersed monkey kidney cells, *Proc. Soc. Exp. Biol. Med.* 85 202.

ZIELKE-TEMME, B. & HOPWOOD, L. (1982) Time-lapse cinemicrographic observations of heated G1-phase Chinese hamster ovary cells, *Radiat. Res.* 92 332–342.